

**Assessing associations between measures of
reduced glomerular filtration rate, abnormal
cardiovascular risk factors, and risk of
cardiovascular morbidity and mortality**

by

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Abstract

End stage renal failure is associated with a massively increased risk of cardiovascular disease, and evidence suggests that this increase in risk begins early in the development of chronic kidney disease. This thesis considers the hypothesis that small reductions in glomerular filtration rate (GFR), across the population range, are associated with a clustering of cardiovascular risk factors and increased risk of cardiovascular disease and death. Existing methods to accurately measure GFR are difficult to perform and unsuitable for large studies, while GFR estimated from blood creatinine or cystatin c concentration is inaccurate.

The performance of a new method of assessing GFR, in which total plasma iothexol clearance is measured using dried capillary blood spots, was examined in a cross-sectional study of 81 consecutive individuals undergoing routine measurement of GFR. The new blood spot iothexol clearance (BSIC) method (using 3 blood spot samples) assessed GFR accurately compared to traditional iothexol clearance using 3 timed plasma samples (mean \pm standard deviation [BSIC - reference method]: 1.1 ± 7.7 ml/min/1.73m²); prediction equations to estimate GFR from blood creatinine and cystatin c concentration performed poorly. The results were similar when 2 blood spots (2 and 4 hours) were used, but using only a single 4 hour blood spot resulted in some loss of accuracy.

The feasibility of using the BSIC method when the blood spot sampling is completed by participants at home was assessed in a cross-sectional study of 111 individuals.

Following a short training, 100% of participants completed satisfactory baseline samples,

97% returned 2 timed samples through the post and 90% found the procedure acceptable. However, 21% returned small or poor quality blood spots and there was statistical evidence of rounding-up of the recorded sampling time. Among 106 participants with measurements of BSIC-GFR, GFR estimated from blood creatinine, and cystatin c concentration, one or more measures of GFR were positively correlated with blood high density lipoprotein cholesterol concentration, and were inversely correlated with blood concentrations of triglyceride, C-reactive protein, fibrinogen, and homocysteine. BSIC-GFR was not more strongly related to cardiovascular risk factors than GFR estimated from blood creatinine concentration (eGFR). GFR estimated from plasma cystatin c concentration was strongly related to measures of body fat, but no relationship was seen with the other GFR measures.

In a meta-analysis of cohort studies assessing the relationship between eGFR and risk of death and cardiovascular events, which included 4 061 003 and 1 372 820 individuals for each outcome respectively, a 30% lower eGFR was associated with a 20-30% increase in risk of each outcome, depending on the type of study examined. However, there was significant heterogeneity between the studies. These overall results may underestimate the impact of lower eGFR among those with chronic kidney disease since, in two large studies together contributing 85% of the deaths, lower eGFR was not associated with increased risk of death until eGFR was below 60 ml/min/1.73m². In one large study contributing 218 000 deaths, the relative risk of death associated with lower eGFR was larger at younger ages, but eGFR had greater absolute impact at older ages and among those with prior vascular disease. Because eGFR is only weakly related to

measured GFR among healthy individuals, these results may underestimate the relationship between 'true' GFR and risk of death and vascular disease.

This work demonstrates an association between measures of GFR and cardiovascular risk factors, and increased risk of vascular disease, although the methods used to assess GFR may have introduced bias. Large scale studies in which GFR is accurately measured are needed. Using the new BSIC method for this purpose is potentially feasible, but further work is required to ensure accuracy when the blood sampling is completed by participants themselves.

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Declaration of the author's contribution to the work

This thesis was composed by the author, however, a number of individuals have contributed to the work presented in this thesis. The contribution of the author to each chapter is outlined in this section. I should also be noted that Professor Colin Baigent and Dr Martin Landray provided guidance throughout this research.

Chapter 1: This chapter, including the relevant literature searches, is entirely the work of the author.

Chapter 2: The laboratory method used to measure the concentration of iohexol in dried capillary blood spots was developed by the South West Thames Institute of Renal Research, St Helier Hospital, Carshalton, and participant recruitment and data collection for this study was undertaken there. The author analysed the data, with assistance from Dr Jonathan Emberson, a statistician at the Clinical Trial Service Unit, University of Oxford.

Chapters 3 and 4: Protocol development, participant recruitment and data collection for this study were conducted by the author, with assistance from administrative staff and a research nurse at the Clinical Trial Service Unit. Data analysis was planned and conducted by the author.

Chapter 5: The author produced the protocol, conducted the literature search and extracted the data for this meta-analysis. Data analysis was planned by the author and Dr Jonathan Emberson, who conducted the analyses.

This work has not been submitted for any other degree or professional qualification.



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List of abbreviations

AUC	Area under the curve
BMI	Body mass index
BSIC	Blood spot iohexol clearance
BSA	Body surface area
CETP	Cholesteryl ester transfer protein
CKD	Chronic kidney disease
CRIB	Chronic Renal Failure in Birmingham study
⁵¹ Cr-EDTA	Chromium-51 ethylene diamine tetra-acetate
CRP	C-reactive protein
EDTA	Ethylene diamine tetra-acetate
ESKD	End stage kidney disease
GFR	Glomerular filtration rate
HDL	High density lipoprotein
HL	Hepatic lipase
HPLC	High performance liquid chromatography
IDL	Intermediate density lipoprotein
IDMS	Isotope-dilution mass spectrometry
LCAT	Lethicin cholesterol acetyltransferase
LDL	Low density lipoprotein
LP	Lipoprotein lipase

LVH	Left ventricular hypertrophy
MDRD	Modification of Diet in Renal Disease study
MI	Myocardial infarction
MSE	Mean squared error
NKF-KDOQI	National Kidney Foundation Kidney Disease Outcomes Quality Initiative
PENIA	Particle-enhanced nephelometric immunoassay
PETIA	Particle-enhanced turbidometric immunoassay
^{99m}Tc -DTPA	^{99m}Tc -diethylene-triamine-penta-acetic acid
SEARCH	Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine
SD	Standard deviation
UK	United Kingdom
VLDL	Very low density lipoprotein

Introduction

End stage kidney disease is associated with increase risk of cardiovascular disease

The risk of cardiovascular death among individuals with end stage kidney disease (ESKD) receiving haemodialysis is between 10 and 100 times that of members of the general population of a similar age (1), and, among such patients, cardiovascular disease is the leading cause of death accounting for about 60% of deaths (2). At least some of the increased cardiovascular risk appears earlier in the natural history of kidney failure since cardiovascular disease is already highly prevalent among individuals starting renal replacement therapy. Among those commencing haemodialysis in the United Kingdom (UK), 22% have a history of ischaemic heart disease (31% of those over 65 years) and around 12% have already suffered a myocardial infarction (3), while 41% of incident haemodialysis patients in the United States have a history of coronary artery disease (4). In a cross-sectional study of nephrology outpatients in the UK with elevated serum creatinine concentrations, 34% had a history of vascular disease (5) and in a similar study in Canada the prevalence of cardiovascular disease was 39% (6). In a prospective study of 147 nephrology outpatients with impaired kidney function in France, the incidence of cardiovascular events was nearly 3 times that seen among members of the general population of the same age and sex (7).

It should be noted, however, that, although individuals with ESKD are at massively increased risk of cardiovascular disease, the pattern of vascular disease seen in these patients is different to that observed in the general population. Routine mortality

statistics from the US suggest that around two thirds of cardiovascular deaths in the general population are attributed to coronary heart disease. In contrast, reports from the United States Renal Data System show that, while 60% of deaths among haemodialysis patients are attributed to cardiovascular disease, of these, only 20% are attributed to acute myocardial infarction and 12% are attributed to stroke, leaving nearly 70% of cardiovascular deaths attributed to 'other cardiac' causes (2). Since these data rely on routine death certification to ascribe cause of death, there is considerable uncertainty about the underlying pathophysiology of these 'other cardiac' deaths. While atherosclerotic coronary artery disease may be a contributing factor in these patients, structural heart disease also appears to be important (8). Among 433 patients assessed by echocardiography at the initiation of dialysis, 74% had left ventricular hypertrophy (LVH) and 15% had impaired systolic function (9) and, among haemodialysis patients, LVH identified by echocardiography is associated with increased risk of death (10). Arterial calcification is substantially increased among dialysis patients compared to the general population (11). Calcification of the arterial media, which commonly occurs in ESKD, may contribute to the development of heart failure by causing arterial stiffness (12). Thus, controversy exists regarding whether ESKD is a pro-atherogenic state or whether the excess risk of cardiovascular death seen in this population is related to factors such as arterial calcification, chronic volume overload and a long exposure to hypertension leading to structural heart disease.

Early stages of chronic kidney disease are defined by reduced glomerular filtration rate

While those with ESKD represent a clearly defined population, study of earlier stages of kidney failure has been limited by difficulty in defining kidney disease (13). Definitions used in epidemiological studies have included: a serum creatinine concentration above a certain cut point (14); individuals under follow-up by a nephrologist (7); and the recording of an appropriate International Classification of Disease code in health care provider records (15). Since clearance of small solutes from the circulation, assessed by the glomerular filtration rate (GFR), is regarded as the principal function of the kidneys, the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF-KDOQI) have issued guidelines in which chronic kidney disease (CKD) is classified according to severity on the basis of GFR (Table 0.1) (16)). However, GFR is difficult to measure directly and therefore, in most settings, GFR is estimated from blood creatinine concentration (reviewed in section 1.3.1.4) (16).

GFR may be is a risk factor for death and cardiovascular disease in unselected populations.

Although studies have demonstrated an excess risk of cardiovascular disease in both ESKD and in ‘pre-dialysis’ renal unit populations, examination of GFR as a risk factor for cardiovascular disease in *unselected populations* is useful for two reasons. First, while ESKD is associated with a massively increased risk, it is a relatively rare condition with a prevalence of around 600 per million adults in the UK (3). If the relationship between GFR and cardiovascular disease is continuous across the population range of

GFR, then a larger proportion of the population at increased risk, who may benefit from cardio-protective interventions, could perhaps be identified. Second, the finding of excess cardiovascular risk among individuals with advanced CKD could be the result of reverse causality in which cardiovascular disease results in renal failure. Prospective studies among healthy populations, in which GFR is measured at baseline and related to future cardiovascular events, are necessary to examine GFR as a *determinant* of cardiovascular disease. Although no population based prospective cohort studies have measured GFR directly, a number of studies have demonstrated a modest association between lower GFR estimated from serum creatinine concentration (eGFR) and an increased risk of death and cardiovascular disease (17-19). However, since eGFR is only weakly related to 'true' GFR in healthy populations (20;21), the results of these studies may distort the true relationship between GFR and cardiovascular risk.

Evidence suggests that lower GFR may be a causal risk factor for vascular disease

Several lines of evidence support the hypothesis that reduction in GFR is a causal risk factor for cardiovascular disease. First, in ESKD the substantial improvement in mortality associated with renal transplantation (22) suggests that, in this population, at least some of the cardiovascular risk is driven by the absence of independent renal clearance function. Second, CKD is associated with a number of adverse cardiovascular risk factors (23), including hypertension (24), a pro-atherogenic lipid profile (25), higher blood concentration of inflammatory markers such as C-reactive protein (26), higher blood homocysteine concentrations (27) and increased oxidative stress (28) (Table 0.2). Anaemia and abnormalities in the metabolism of calcium and phosphate are common in

CKD (29;30) and may contribute to the development of structural heart disease (12;31). Third, in animal studies loss of GFR results in dyslipidaemia (32), hypertension (33) and accelerated cardiovascular disease (34;35).

This thesis considers the hypothesis that small reductions in GFR, across the range of GFR observed in the general population, are associated with a clustering of cardiovascular risk factors which subsequently result in an increased risk of cardiovascular disease. In addressing this hypothesis, this thesis will first consider how GFR can best be measured in large scale epidemiological studies, before examining how measures of GFR are related to cardiovascular risk factors and risk of cardiovascular mortality and morbidity.

Table 0.1: NKF-KDOQI classification of chronic kidney disease

Stage	Description	GFR <i>ml/min/1.73m²</i>
1	Kidney damage with normal or increased GFR	≥90
2	Kidney damage with mildly decreased GFR	60-89
3	Moderately decreased GFR	30-59
4	Severely decreased GFR	15-29
5	Kidney failure	<15 or dialysis

Chronic kidney disease is defined as either kidney damage or GFR (glomerular filtration rate) <60 ml/min/1.73m² for ≥ 3 months. Kidney damage is defined pathological abnormalities or markers of damage including abnormalities in blood or urine tests or imaging studies

Reproduced from the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF-KDOQI) clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification (16)

Table 0.2: Known and suspected cardiovascular risk factors associated with chronic kidney disease

Risk factor	Association with CVD in general population studies		Association with CKD	
	Observational association with CVD	Causal relationship confirmed by RCTs	Change in CKD stage 5	Change in CKD stages 2-4
Blood pressure	✓ (36)	✓ (37;38)	↑ (9)	↑ (5;39)
Blood lipids				
LDL-Cholesterol	✓ (40;41)	✓ (42)	↔ (43;44)	↔ (5)
Apolipoprotein B	✓ (45)	-	↔ (46)	↔ (47)
HDL-Cholesterol	✓ (48)	-	↓ (49)	↓ (5)
Apolipoprotein A ₁	✓ (45)	-	↓ (46)	↓ ↔ (46;47)
Triglycerides	✓ (50)	-	↑ (43)	↑ (5)
Inflammatory markers				
C-reactive protein	✓ (51)	-	↑ (52;53)	↑ (54)
Fibrinogen	✓ (55)	-	↑ (56)	↑ (54)
Interleukin-6	✓ (57)	-	↑ (58)	↑ (59)
Markers of endothelial dysfunction				
Von Willebrand factor	✓ (60)	-	↑ (61)	↑ (54)
ADMA	✓ (62)	-	↑ (63)	↑ (64)
Other				
Lipoprotein (a)	✓ (65)	-	↑ (66;67)	↑ (68)
Homocysteine	✓ (69)	-	↑ (27)	↑ (5;70)

CVD, cardiovascular disease; CKD, chronic kidney disease (16); RCT, randomized controlled trial; LDL, low density lipoprotein; HDL, high density lipoprotein; ADMA, asymmetric dimethyl-arginine

Chapter 1: Measurement of glomerular filtration rate

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1.1 Introduction

Glomerular filtration rate (GFR), defined as the volume of fluid filtered from the renal glomerular capillaries into Bowman's space per unit of time, is regarded as the principal measure of kidney function (16). This thesis is concerned with the relationships among GFR, cardiovascular risk factors, and cardiovascular disease; how these relationships might be affected by the method used to measure GFR; and the development of an accurate GFR method suitable for large scale epidemiological studies. Before considering these issues, this chapter describes existing methods for measuring GFR, the suitability of these methods for large scale studies, and potential sources of error. Since this thesis examines the relationship between GFR and cardiovascular risk in adults, the measurement of GFR in children is not discussed here.

GFR is equivalent to the urinary clearance of a tracer substance provided that the tracer is physiologically inert, is freely filtered at the glomerulus, and is neither secreted nor reabsorbed by the renal tubules (71). Since the 1930s, the gold standard method of assessing GFR has been the measurement of renal inulin clearance using multiple timed urine and blood samples collected while administering an intravenous inulin infusion along with oral, or intravenous, fluids to ensure constant urine flow rates (72). Since then a number of techniques have been developed to assess GFR from the appearance of exogenous tracer substances in the urine, or the disappearance of such substances from the plasma. In this thesis, these methods are referred to as 'direct GFR methods'. More

recently, methods have been developed to estimate GFR from a single measurement of the plasma concentration of endogenous substances which are filtered by the glomerulus, referred to here as 'indirect GFR methods'.

1.2 Direct GFR methods

The 'direct GFR methods' vary in terms of the clearance calculation method (urinary clearance or total plasma clearance), the choice of GFR tracer substance and the way in which that substance is administered (continuous intravenous infusion, bolus intravenous injection or subcutaneous injection).

1.2.1 Clearance calculation methods

1.2.1.1 Urinary clearance methods

Urinary clearance of a GFR tracer compound can be calculated from a timed urine collection and appropriate plasma sample using the classic formula (71);

a) $\text{Clearance (ml/min)} = U \times V / P \times t$

(Where U is the concentration of the tracer in the urine, V is the volume of urine in ml, P is the plasma concentration of the tracer and t is the number of minutes for which the urine was collected).

If the tracer has been administered by continuous infusion, the plasma sample may be taken at any time during the urine collection period. If a bolus injection of tracer has been given, the plasma sample can be collected at the mid point of the timed urine collection or an average of plasma tracer concentrations measured at the beginning and

end of the urine collection period can be used. Potential sources of error in this method include incomplete bladder emptying and incorrect timing of the plasma or urine samples, along with laboratory variation in the measurement of the concentration of the tracer blood and urine. In order to minimize the potential effect of incomplete bladder emptying, traditional GFR protocols require the insertion of a urinary catheter (although, more recently, this has been reserved for individuals in whom bladder ultrasound demonstrates a residual urinary volume (73)). Errors can be reduced by collecting several timed urinary and blood samples and taking an average of the results since considerable variability is seen between serial clearance periods during the same test. In the Modification of Diet and Renal Disease study in which renal clearance of 125 -Iothalamate was measured in around 1600 participants enrolled in the baseline assessment, 4 clearance periods were used. The coefficient of variation among the 4 clearance periods was 9.4% (73).

1.2.1.2 Total plasma clearance methods

Following a single injection, clearance of an exogenous tracer substance from the plasma can be estimated from the dose of administered tracer compound and the area under the plasma disappearance curve (AUC) according to the formula (74);

b) $\text{Clearance} = \text{dose} / \text{AUC}$

Provided the tracer substance has little or no extra-renal clearance, clearance from the plasma is approximately equivalent to GFR.

Following an intravenous injection, the plasma concentration of GFR tracers fall exponentially as the tracer diffuses into the extra-vascular space (the rapid equilibration phase) and thereafter declines linearly (75). Complete equilibration between the plasma and the extra-vascular space takes 2-8 hours depending on the volume of distribution of the tracer compound and the size of the individual (75;76). Direct measurement of the AUC requires a large number of plasma samples, but the area under the curve can be estimated from 2 or 3 plasma samples by first assuming a one compartment model using formula c.

$$c) \quad \text{Clearance}_{(1)} = \text{Dose} / (C_0 / k)$$

(where C_0 is the intercept and k the slope of the least squares regression line of Ln [plasma tracer concentration] against time).

Then a mathematical adjustment can be made to account for the rapid equilibration phase using the Brochner-Mortensen (77) formula;

$$d) \quad \text{Clearance}_{(\text{final})} = 0.990778 \times \text{Clearance}_{(1)} - 0.001218 \times \text{Clearance}_{(1)}^2$$

In order to simplify the procedure further, a number of formulae have been developed to estimate total plasma clearance using just one plasma sample (78). These methods use an estimate of the volume of distribution and the administered dose of the tracer substance as a surrogate for the tracer concentration in the blood at time zero. The most commonly used one sample method, developed by Jacobsen, is described in the following formulae (79);

$$e) \quad \text{Clearance}_{(1)} = (\text{Ln} [\text{dose} / C_t \times V]) / (0.0016 + t/V)$$

(where t = sample time, C_t = tracer concentration at time t , V = volume of distribution of tracer).

This estimate is then adjusted by a factor, 'm', which is dependent on the initial clearance value.

f) $m = 0.991 - 0.00122 \times \text{Clearance}_{(1)}$

g) $\text{Clearance}_{(\text{final})} = (\text{Ln} [\text{dose} / C_t \times V/m]) / (0.0016 + mt/V)$

Potential sources of error for all plasma clearance methods include the administration of an incorrect dose of tracer (some protocols advocate weighing the syringe containing the tracer prior to injection to ensure exact measurement of dose), incorrect timing of plasma samples and extra-renal elimination of the tracer, along with laboratory variation in the plasma tracer assay. In conditions such as ascites, which are associated with marked alterations in the distribution of fluid within the body, the rapid elimination phase may be different to that assumed in the Brochner-Mortensen method, resulting in additional error when this calculation method is used (80). In addition, among individuals with very low GFR, a late (usually 24 hour) sample is required in order to accurately estimate the area under the plasma curve (81). Since the one sample methods estimate the volume of distribution of the marker substance from anthropomorphic measurements such as weight (78;79), variation in body composition could result in additional error.

1.2.2 Method of tracer administration

The classical inulin clearance protocol requires the administration of an intravenous infusion to establish constant plasma inulin concentrations (72). However, administration of a single intravenous injection is a simpler and more practical procedure for patients and staff and is widely used with both total plasma clearance (82-84) and renal clearance techniques (85;86). Subcutaneous injection of tracer compound has also been used in conjunction with renal clearance techniques (85).

1.2.3 Choice of exogenous GFR tracer

Although the fructose polymer inulin has been used for the assessment of GFR since the 1930s (72), the relatively high cost and time consuming chemical analysis techniques have prompted the use of alternatives tracers. Radio-labelled markers such as chromium-51 ethylene diamine tetra-acetate ($^{51}\text{Cr-EDTA}$), $^{99\text{m}}\text{Tc}$ -diethylene-triamine-penta-acetic acid ($^{99\text{m}}\text{Tc-DTPA}$) or ^{125}I -iothalamate also fulfill the criteria for a GFR tracer and, being relatively easily measured using a scintillation counter, have been used for this purpose since the 1960s (82;87;88). However, the strict procedures required for safe handling, storage and disposal of radio-isotopes are a major disadvantage in the use of these compounds. More recently, both ionic (e.g. iothalamate) and non-ionic (e.g. iohexol or iopromide) contrast media have been used to measure GFR (76). The plasma concentration of these agents can be reliably measured by high performance liquid chromatography (HPLC) (89) or X-ray fluorescence analysis (90); although the later method is less accurate when small volumes of tracer are administered (91). Ionic and, to a lesser extent, non-ionic contrast media have been associated with adverse reactions

during angiography or radiological studies (typical dose of about 100ml) (92;93), although data suggest that adverse reactions are very rare when small doses of around 5ml are used (94). The characteristics of the individual GFR tracer compounds are summarized in table 1.1.

1.2.4 Adjustment for body surface area

GFR is directly related to intravascular volume, and therefore body size (71). To allow meaningful comparison between individuals, GFR is usually standardised to a body surface area (BSA) of 1.73 m². Body surface area is difficult to measure directly, and is usually calculated from measurements such as height and weight using a prediction equation such as the commonly used formula by Dubois and Dubois (95);

$$BSA = 0.20247 \times \text{height (m)}^{0.725} \times \text{weight (kg)}^{0.425}$$

1.2.5 ^{99m}Tc-diethylene-triamine-penta-acetic acid (^{99m}Tc-DTPA) renography

A less invasive method of measuring renal ^{99m}Tc-DTPA clearance has been proposed, in which the uptake of ^{99m}Tc-DTPA in both kidneys measured by renography 2-3 minutes after an intravenous injection is used to estimate GFR (96). This method does not require urine or blood sampling, and can be completed in a short time period. Unfortunately, when compared to total plasma ^{99m}Tc-DTPA clearance (97) and ⁵¹Cr-EDTA clearance (98), and renal clearance of inulin (99), ^{99m}Tc-DTPA renography has very poor accuracy.

1.2.6 Use of direct GFR methods in epidemiological studies

Direct methods of assessing GFR are difficult to perform and time consuming, usually requiring that the participant spend at least 4 hours at the hospital, undergo multiple venepuncture and, in some protocols, collect timed urine collections which may require bladder catheterisation. When radio-labelled GFR tracers are used, strict protocols, which often include restrictions for participants after the test is completed, are required to ensure the safety of staff, participants and the general public. Thus, using existing methods, direct measurement of GFR in a large scale population based epidemiological study would be impractical.

In addition, because of short term changes in true GFR and measurement error, there is considerable intra-individual variability in directly measured GFR. For example, among individuals enrolled in the Modification of Diet in Renal Disease study in whom renal clearance of 125 -Iothalamate was measured 3 months apart, the median coefficient of variation was 6.3% (73).

1.3 Indirect GFR methods

Plasma concentrations of endogenous substances which are filtered by the glomerulus, such as creatinine or the low molecular weight protein, cystatin c, are inversely proportional to GFR: Therefore, GFR can be indirectly estimated from single measurements of the blood concentration of these 'GFR markers'.

1.3.1 Blood creatinine as a marker of GFR

The observation that creatinine is eliminated from the body by glomerular filtration led to the development of creatinine clearance as a measure of GFR in the 1930s (100).

Since then, measurement of blood creatinine concentration has become the most commonly performed assessment of GFR. The physiology of creatinine metabolism, and methods used to assess GFR from blood creatinine concentration are described below.

1.3.1.1 Creatinine production

Creatinine has no known biological function and is generated by spontaneous degradation of creatine and phosphorylcreatine (figure 1.1) (101). Creatine is synthesised in liver, kidneys and pancreas from arginine by 2 enzymically mediated reactions; first, the conversion of arginine and glycine to guanidinoacetate and ornithine, and then the methylation of guanidinoacetate to form creatine (figure 1.1) (101).

Creatine is then transported to muscle, and other tissues, where it is phosphorylated to form the high energy compound phosphocreatine, which plays an important part in muscle contraction (101). About 2% of total body creatine, which is predominantly located in muscle, spontaneously converts to creatinine each day (75). Creatinine

production, therefore, is heavily dependent on muscle mass, but remains relatively constant within an individual over the short term. Dietary intake of meat can, however, result in an increase in blood creatinine concentration and 24 hour creatinine excretion of 10-20% (102;103).

1.3.1.2 Creatinine elimination

Creatinine is freely filtered by the glomerulus and therefore blood creatinine concentration is inversely proportional to GFR (104) while urinary creatinine clearance correlates with GFR (100). However, since small amounts of creatinine are excreted by the renal tubules, creatinine clearance generally exceeds inulin clearance by 10-20% (100;104). The ratio between creatinine and inulin clearance is considerably higher among individuals with substantially reduced GFR (104). Among individuals with very high GFR, tubular re-absorption of creatinine may lead to an underestimation of GFR by creatinine clearance (105). Cimetidine, which inhibits tubular creatinine secretion, is sometimes administered as part of a creatinine clearance protocol (104).

In advanced chronic kidney disease (CKD), blood creatinine concentrations are substantially lower than that predicted by a reciprocal relationship with GFR, as a result of both increased tubular creatinine secretion and extra-renal removal of creatinine from the circulation. In stage 5 CKD ($\text{GFR} < 15 \text{ ml/min/1.73m}^2$ (16)), between 16 and 66% of generated creatinine disappears from the body by mechanisms other than excretion in the urine or faeces (101). This 'creatinine deficit' is thought to be the result of increased

degradation of creatinine either by 'creatininase' producing gut bacteria or by oxidative creatinine degradation pathways (101).

1.3.1.3 Measurement of creatinine concentration in blood and urine

Creatinine concentration in plasma, serum or urine can be measured using an alkaline picrate ('Jaffé') reaction, enzymatic methods, high performance liquid chromatography (HPLC) or isotope-dilution mass spectrometry (IDMS) methods. The concentration of creatinine in plasma and serum is roughly equivalent and is used interchangeably (106).

Originally described by Jaffé in 1886 (107), the reaction between creatinine and picrate ions which, under alkaline conditions, results in an orange-red complex, forms the basis of creatinine estimation in most routine laboratories. This reaction is not specific for creatinine as a number of substances also undergo the Jaffé reaction (e.g. glucose, acetone, cephalosporins, pyruvate) (100;108). A large number of methods, involving kinetic assays and the addition of variety of specific reagents, have been developed aimed at reducing interference from these non-creatinine chromogens (109) although interference from such substances can still lead to overestimation of blood creatinine concentration by as much as 15-25% depending on the specific reagents used (108).

In order to overcome the lack of specificity associated with the Jaffé based assays, a number of multi-step enzymatic methods have been developed (100;108). Although more specific than the modified Jaffé methods, interference from non-creatinine substances can still occur with enzymatic methods (110), and their greater cost and longer run times have limited the use of these methods in clinical practice. A number of

HPLC methods for measurement of creatinine have been developed which, although rarely used in clinical practice, are more specific than conventional methods (108).

IDMS methods, which have very high specificity and precision, have been developed for measurement of creatinine in reference materials, but are available in only a few specialized laboratories worldwide (108).

Considerable variation exists between laboratories in the measurement of creatinine. A survey of 5624 laboratories found that the 50 instrument-method combinations used measured creatinine with a bias of -5.3 to 27.4 $\mu\text{mol/l}$ compared to the reference laboratory IDMS method (111). A program to standardize all laboratory creatinine methods to an IDMS reference method has been proposed (108). However, although such standardisation would remove the bias associated with some creatinine methods or instruments, lack of specificity could still result in erroneous measurement of serum creatinine concentration for an individual. Inter-laboratory quality-control programs suggest that within laboratory coefficients of variation are between 2 and 8.5% at a blood creatinine concentration of 88.4 $\mu\text{mol/l}$ for most creatinine assays (108).

Creatinine is stable for up to 4 days in whole blood stored at 4 °C, but each day at which whole blood remains at room temperature increases subsequent measurement of plasma concentration by about 5% (112). The concentration of creatinine concentration in serum stored at -80 °C is unaffected by thawing and refreezing the sample (113).

1.3.1.4 Estimation of GFR from blood creatinine concentration

Since GFR is approximately proportional to the reciprocal of blood creatinine concentration, mathematical formulae have been used to predict GFR from the concentration of creatinine in the blood. These formulae also include variables such as weight, height, age or sex, which are related to muscle mass and so correlate with blood creatinine concentration. Published formulae to estimate GFR from blood creatinine concentration are shown in table 1.2. However, this thesis will principally consider the 2 most commonly used formulae; the Cockcroft Gault formula and the abbreviated Modification of Diet in Renal Disease (MDRD) formula.

The abbreviated MDRD formula (table 1.2) was developed to predict body surface area normalised renal clearance of ^{125}I -iothalamate among 1628 individuals with CKD screened for the Modification of Diet in Renal Disease study who had a mean GFR of 40 ml/min/1.73 m² (114). Studies assessing the performance of the MDRD formulae are summarized in table 1.3.

The Cockcroft Gault formula (table 1.2) was developed to predict creatinine clearance, without adjustment for body surface area, among male hospital inpatients with a mean creatinine clearance of 73 ml/min/1.73m² (115). The estimate must, therefore, be adjusted for body surface area to predict body surface area normalised GFR. Since creatinine clearance generally overestimates GFR because of tubular creatinine secretion, it has been suggested that the estimate produced by the Cockcroft Gault formula multiplied by a factor of 0.85 better reflects GFR (116) although, in practice, this

adjustment is often not made. Studies assessing the performance of the Cockcroft and Gault formula are summarised in table 1.4.

1.3.2 Creatinine clearance as a measure of GFR

Creatinine clearance, calculated according to formula 'a' in section 1.2.1, has been used as a measure of GFR since the 1930s (100). Typically, urine is collected for 24 hours and a blood sample is taken during this period. Sources of error in this method include inter-individual variability in tubular creatinine secretion and, more importantly, failure to accurately collect a precise timed urine collection. A large number of studies have confirmed that, as a result of these problems, measured creatinine clearance does not represent an advantage over GFR estimated from a single measurement of blood creatinine concentration in terms of accuracy (117-127), while representing a substantial disadvantage in terms of convenience.

1.3.3 Cystatin C as a marker of GFR

Cystatin c, also known as gamma trace protein, was proposed as an alternative to creatinine as an endogenous marker of GFR in the 1980s (128) and since then a number of studies have suggested that blood concentration of cystatin c has better diagnostic accuracy than blood creatinine concentration (129;130).

1.3.3.1 Physiological functions of cystatin c

Cystatin c is a low molecular weight protein and a potent inhibitor of the papain type cysteine protease enzymes, known as cathepsins (75). The cathepsins are part of a broader group of protease enzymes which also include the serine proteases, matrix metalloproteases and the aspartate proteases. They are most stable at an acidic pH and are active within lysosomes where they break down unwanted proteins; however they can also act outside lysosomes and are involved in a number of specific physiological areas. Cathepsins K, S and L have been shown in vitro to degrade various components of the intracellular matrix, such as elastin, laminin, fibronectin and type I collagen (131;132). Cathepsin K is produced by osteoclasts and is involved in bone remodelling (133). Cathepsin S is involved in the intracellular trafficking of MHC class II molecules and therefore regulation of antigen presentation (134). The various cathepsins are differentially expressed in different cell types and their expression and secretion in experimental cell lines can be induced by various cytokines (135). Cystatin c is expressed by all nucleated cells and the gene promoter is of a housekeeping type (136). However, in experimental cell lines, the expression and secretion of cystatin c can be

effected by inflammatory cytokines (135), steroid hormones (137) and lipopolysaccharide (138). Cystatin c has also been shown to antagonise TGF- β by a cathepsin independent mechanism suggesting that it may have physiological functions other than cathepsin inhibition (139).

1.3.3.2 Cystatin c production

While few studies have directly assessed the determinants of cystatin c production, a number of factors, other than GFR, are related to an individual's usual long term blood cystatin c concentration. Mean plasma cystatin c concentration among healthy non smoking individuals under 65 is around 0.7 ± 0.1 mg/l, with slightly higher concentration in men than women (140). In a large community sample, smokers and those with greater body weight had higher plasma cystatin c concentrations, after adjustment for measured creatinine clearance (141). Six cystatin c gene polymorphisms have been identified which are related to blood cystatin c concentration (142;143). In one study, each of the mutant alleles (82C,78G,5A,4C) was associated with a lower cystatin c concentration (142). There was linkage disequilibrium and a mutant haplotype (82C,78G,5A,4C) was associated with 0.03-0.04 mg/l lower concentration of cystatin c per copy. In this study of 155 Swedes, ~35% were heterozygous and ~6% were homozygous for this mutant haplotype. In another study (143), for each of the mutant alleles (148G, 82C,5A,4C) one copy was associated with about 0.04-0.1 mg/l lower cystatin c concentration. A mutant haplotype (82C, 5A,4C) was associated with ~0.06 mg/l lower blood cystatin c concentration per copy. In this study of 1013 patients with coronary heart disease, ~4% were homozygous and 31% heterozygous for this mutant haplotype.

1.3.3.3 Short term changes in blood cystatin c concentration

Plasma concentration of cystatin c is increased in hyperthyroidism and decreased in hypothyroidism based on serial measurements in patients with thyroid disorders at presentation and after recovery (144;145). The magnitude of the change in blood cystatin c concentration in these studies was around 0.2mg/l for both hypo- and hyperthyroidism. Subclinical hypo- and hyper- thyroidism is associated with a smaller change in blood cystatin c concentration of around 0.1 mg/l (146). Several studies have shown an increase in blood cystatin c concentration following the administration of large doses of exogenous steroids (147-149) but none have adjusted for measured GFR.

Only four small clinical studies have assessed serial cystatin c measurements in response to acute vascular disease or other systemic insult and none of these measured GFR. Noto *et al* (150) measured blood cystatin c and creatinine concentration in 61 patients presenting with acute myocardial infarction (MI), 61 patients presenting with acute coronary syndrome without confirmed MI and 61 healthy volunteers. Mean cystatin c concentration was 0.05 mg/dl lower in those presenting with acute MI than the healthy controls but subsequently increased. The cystatin c to creatinine ratio in cases of MI was 1.02 at admission a 1.14 prior to hospital discharge. Gupta-Malhotra M *et al* (151) found that serum cystatin c concentration was lower among 17 children presenting with acute Kawasaki's disease compared to control children and that the serum cystatin concentration increased after treatment of the disease. Two studies reported a fall in

cystatin C concentration in the first 24 hours following total hip replacement (152) and hysterectomy (153). However both studies were small and neither paper report the quantity of intravenous fluid administered. There is no evidence of variation in plasma cystatin c levels with circadian rhythm (154).

1.3.3.4 Elimination of cystatin c

Clearance of cystatin c from the circulation is almost entirely through glomerular filtration (155). Once filtered, cystatin c undergoes complete proteolysis within the tubular cells (155). Plasma cystatin c concentrations are therefore inversely proportional to GFR (156), but assessment of GFR from the urinary clearance of cystatin c is not possible.

1.3.3.5 Measurement of cystatin c concentration in plasma and serum

Plasma concentration of cystatin c is measured by immunoassay, most commonly either by Particle-Enhanced Turbidometric Immunoassay (PETIA) or by Particle-Enhanced Nephelometric Immunoassay (PENIA) (75). Some studies have suggested that PENIA assays have better reproducibility at higher plasma cystatin c concentrations (157) and are less susceptible to interference from other substances such as bilirubin (158) than the PETIA assays. Plasma cystatin c concentrations are similar, or very slightly lower (3%), in serum than plasma (156;157;159). There is currently no standardisation of cystatin c methods between laboratories.

Serial measurements in healthy volunteers with repeated analysis of samples demonstrate that the coefficient of variation attributable to biological variation is around

13%, while that attributable to analytical variation is around 9% (160). Blood cystatin c concentrations are stable in whole blood stored at room temperature for up to 4 days [personal communication, Clinical Trial Service Unit laboratory].

1.3.3.6 Estimation of GFR from blood cystatin c concentration

A number of formulae have been developed to predict GFR from blood cystatin c concentration (table 1.5). As blood cystatin c concentration is less dependent on muscle mass than blood creatinine concentration (140), these formulae mostly involve only constant terms. All the formulae, except for one (161), were developed to predict GFR standardised to 1.73m² body surface area. Studies assessing the performance of cystatin c formulae are summarized in table 1.6. In one study GFR estimated from blood concentrations of both cystatin c and creatinine was more closely related to the reference GFR than estimates using each marker alone (r^2 of 0.891 for the equation including both markers compared to 0.825 for the MDRD formula and 0.852 for a cystatin c based formula) (162).

1.3.4 *Other endogenous GFR markers*

A number of other endogenous GFR markers have been proposed, including other low molecular weight proteins and urea. Urea, a nitrogen-containing by product of protein metabolism, is predominantly cleared from the circulation by the kidneys and the concentration of urea in serum or plasma can easily quantified by most laboratory auto-analysers (75). However, significant ‘back diffusion’ of filtered urea from the renal tubules into the circulation occurs and, more importantly, blood concentrations of urea

are substantially affected by non-renal factors such as diet and hepatic synthesis limiting its use as a GFR marker (75).

Beta-2-microglobulin is associated with the histocompatibility antigen complex on the surface of nucleated cells and is shed from cells during cellular turnover. Like cystatin c, β 2-microglobulin is removed from circulation by glomerular filtration and degraded in the proximal tubules (75). Blood beta-2-microglobulin concentrations are inversely proportional to GFR (128) and show similar diagnostic accuracy to blood cystatin c or creatinine concentrations (163;164). However, blood concentrations of β 2-microglobulin are massively elevated in a number of haematological malignancies and, to a lesser extent, in acute inflammation (165) limiting its use as a GFR marker. Another low molecular weight protein, retinol-binding protein, has been proposed as a GFR marker, but plasma concentrations correlate poorly with directly measured GFR (128;163).

1.3.5 Use of indirect GFR methods in epidemiological studies

Since indirect estimation of GFR requires only a single blood sample, it can be easily undertaken in large scale epidemiological studies, and can be retrospectively analysed in stored blood from existing cohorts (166). Cystatin c is stable for several days in whole blood at room temperature and is therefore suitable for use in population surveys in which blood samples are returned to the research centre by post. These methods, therefore, have substantial practical advantages for large scale epidemiology. However, there are a number of disadvantages associated with their use in this setting.

First, the GFR estimates produced by creatinine based prediction equations are critically dependent on calibration of the creatinine assay (167) (section 1.3.1.3). Calibration differences which are relatively constant across the range of blood creatinine concentration have a greater impact on estimated GFR at higher GFR levels when the calibration difference represents a greater proportion of the creatinine value (167). Creatinine assay calibration can have substantial effects on the estimated prevalence of chronic kidney disease (CKD) (16) classified by estimated GFR: In the 3rd National Health and Nutrition Examination Survey, a large population survey in the United States, the prevalence of CKD (defined as GFR estimated by the MDRD formula of $<60\text{ml/min}1.73\text{m}^2$) among non-diabetic adults was 13% (168). This figure fell to 4% assuming a $17.7\text{ }\mu\text{mol/l}$ over-estimation of blood creatinine values (168) while in fact the laboratory used for this study measured blood creatinine concentration $20\text{ }\mu\text{mol/l}$ higher than the laboratory used to develop the MDRD formula (167). Such laboratory calibration differences make comparison of the prevalence and associations of reduced eGFR between study populations unreliable. The use of a revised version of the MDRD formula for use with creatinine assays which have been calibrated to a isotope-dilution mass spectrometry method may help to overcome these problems in future studies (table 1.2) (169). Laboratory methods also have substantial effect on GFR estimates calculated from blood cystatin c concentration (161) but, since this assay is only used in clinical practice in a few centres, standardisation between laboratories has not been addressed.

Second, the MDRD, Cockcroft-Gault and cystatin c equations have not been validated in some populations, such as individuals from the Indian subcontinent, making comparisons of the impact of absolute eGFR values between ethnic groups difficult.

Third, and most importantly, for an individual, GFR estimated from blood creatinine concentration and cystatin c can be highly discrepant from 'true' GFR (Tables 1.3, 1.4 and 1.6). In an epidemiological study in which these equations are used to assess the relationship between GFR and a particular outcome, this would tend to bias the results towards the null (170).

Fourth, the inclusion of demographic variables, such as age, sex and weight, in the creatinine-based predictions has implications for the use of these equations in research studies. Bias may be introduced when estimated GFR is related to variables which are included in the equation (171). For example, in a large cross-sectional study, a strong relationship was found between weight and GFR estimated by the Cockcroft-Gault formula, when little or no relationship was observed between weight and GFR estimated from the MDRD formula or measured creatinine clearance (171). In cohort studies where analyses are adjusted for age, sex and other variables, the effect of this 'colinearity' is likely to be small. However, in such analyses, GFR estimated from blood creatinine concentration by standard prediction equations does not provide any more information than serum creatinine concentration. Since GFR can be estimated from blood cystatin c concentration with similar accuracy to the creatinine based prediction equations but without inclusion of demographic variables in the formula, this may provide more useful information for epidemiological studies.

Fifth, non renal influences on the concentration of an endogenous marker may introduce specific biases. For example, since creatinine production is directly related to muscle mass (101), loss of muscle mass during chronic illness may be associated with low creatinine concentrations. Among elderly individuals, a J shaped relationship is observed between blood creatinine concentration and non-vascular mortality where individuals with both high and low blood creatinine concentration are at increased risk (172). This J shaped curve could represent an association between low muscle mass and increase risk of non-vascular death, and is not observed with blood concentration of cystatin C (172), which less influenced by body composition than serum creatinine concentration (140).

Thus, the use of indirect GFR methods in epidemiological studies is associated with a number of problems, of which the most limiting is the weak relationship observed between estimated GFR and 'true' GFR among healthy individuals (173;174).

Chapter 1

Tables and figures

Table 1.1: Characteristics of commonly used GFR tracers

Tracer	Extra-renal elimination	Measurement methods	Storage and transport requirements	Disadvantages
Inulin	2 ml/min/1.73m ² (76)	Enzymic (75) HPLC (175)	Immediate separation of plasma and freezing Transport of frozen plasma or local analysis	High cost of tracer Time consuming analysis Limited availability of assays
⁵¹ Cr-EDTA	4 ml/min/1.73m ² (176)	Scintillation counter or gamma spectroscopy (82)	Immediate analysis	Handling of radio-active material
^{99m} Tc-DTPA		Scintillation counter or gamma spectroscopy (87)	Immediate analysis	Handling of radio-active material
¹²⁵ Iothalamate	10 ml/min/1.73m ² (177)	Scintillation counter or gamma spectroscopy (85)	Immediate analysis	Handling of radio-active material
Iohexol	2 ml/min/1.73m ² (76)	HPLC (89;91)	Immediate separation of plasma and freezing Transport of frozen plasma or local analysis Immediate analysis	Limited availability of assays Adverse reactions

HPLC, high performance liquid chromatography; ⁵¹Cr-EDTA, chromium-51 ethylene diamine tetra-acetate; ^{99m}Tc-DTPA, ^{99m}Tc-diethylene-triamine-penta-acetic acid.

Table 1.2: Published formulae to estimate GFR from blood creatinine concentration in adults

Author, year	Population	Formula
Baracksay, 1997 (179)	Elderly	$GFR^{\dagger} = 88 + 0.54 \times 9091 / Cr [\mu mol/l] - 1.06 \times age$
Bjornsson, 1979 (180)	In-patients	$CrCl^* = (25 - 0.175 \times age \times weight \times 0.07) / Cr$ (Female) $CrCl^* = (27 - 0.173 \times age \times weight \times 0.07) / Cr$ (Male)
Cockcroft Gault, 1976 (115)	In-patients	$CrCl^* = (140 - age) \times weight \times (0.85 \text{ if female}) / (72 \times Cr)$
Gates, 1985 (181)	In-patients	$CrCl^* = \text{Female: } 60 \times Cr^{-1.1} + (56 - age) \times 0.3 \times Cr^{-1.1}$ $\text{Male: } 89.4 \times Cr^{-1.2} + (55 - age) \times 0.447 \times Cr^{-1.1}$
Jelliffe, 1973 (182)	Not available	$CrCl^* = (98 - 0.8 \times (age - 20)) / (Cr \times 0.9 \text{ if female})$
Mawer, 1972 (183)	Oncology patients	$CrCl^* = weight \times (25.3 - 0.175 \times age) \times (1 - 0.03 \times Cr) / (14.4 \times Cr \times 70 / weight)$ (Female) $CrCl^* = weight \times (29.3 - 0.203 \times age) \times (1 - 0.03 \times Cr) / (14.4 \times Cr \times 70 / weight)$ (Male)
Mayo, 2004 (20)	Healthy + CKD	$GFR^{\dagger} = \text{Exp}(1.911 + 5.249 / Cr - 2.114 / Cr^2 - 0.00686 \times age - 0.205 \text{ (if female)})$
MDRD, 1999 (116)	CKD	$GFR^{\dagger} = 170 \times Cr^{-0.999} \times age^{-0.176} \times [0.762 \text{ if female}] \times [1.180 \text{ if black}] \times SUN^{-0.170} \times alb^{0.318}$
Abbr. MDRD, 2000 (114)	CKD	$GFR^{\dagger} = 186 \times Cr^{-1.154} \times age^{-0.203} \times [0.742 \text{ if female}] \times [1.210 \text{ if black}]$
IDMS MDRD, 2006 (169)	CKD	$GFR^{\dagger} = 175 \times Cr^{-1.154} \times age^{-0.203} \times [0.742 \text{ if female}] \times [1.210 \text{ if black}]$
Nankivell, 1995 (184)	Renal transplant	$GFR^{§*} = 6.7 / Cr [\mu mol/l] + weight / 4 - SUN (mmol/l) / 2 - 100 / height^2 + [25 \text{ if male or } 35 \text{ if female}]$
Salazar, 1988 (185)	Obese patients	$CrCl^* = (146 - age) \times (0.287 \times weight + 9.74 \times height^2) / (60 \times Cr)$ (Female) $CrCl^* = (137 - age) \times (0.285 \times weight + 12.1 \times height^2) / (51 \times Cr)$ (Male)

GFR, glomerular filtration rate; Cr, Creatinine; CrCl, creatinine clearance; CKD, chronic kidney disease; MDRD, Modification of Diet in Renal Disease study; SUN, Serum urea nitrogen; Alb, serum albumin concentration; Abbr., abbreviated; IDMS, isotope-dilution mass spectrometry.

Unless otherwise indicated units are mg/dl for blood creatinine concentration (to convert mg/dl to $\mu mol/l$ multiply by 88.4), years for age, kg for weight, mg/dl for SUN (to convert mmol/l to mg/dl multiply by 2.801), g/dl for serum albumin concentration (to convert g/l to g/dl multiply by 0.1), and metres for height.

[†]125Iothalamate clearance, * Not adjusted for body surface area, § ^{99m}Tc-diethylene-triamine-penta-acetic acid clearance.

Table 1.3: Studies which assess the performance of an MDRD formula compared to a reference method GFR

First author, publication year	n	Population	Age	GFR ml/min/1.73m ²	Reference method	Formula	Bias (SD) ml/min/1.73m ²	p30% %	p50% %	r ²
Schüek O (186), 2005	79	CKD	20-65 ^a	19±10	Inulin	MDRD	3±4.5	50	75	0.80
Poggio E (174), 2004	828	CKD	56±16	32±28	¹²⁵ Iothalamate	aMDRD	-1	71	89	0.81
Froissart M (21), 2005	1051	Routine GFR <60 ml/min/1.73m ²		34 ^b	⁵¹ Cr-EDTA	aMDRD	1±8.5	83	93	
Rule A (20), 2004	320	CKD	53±15	48±25	Iothalamate	aMDRD	-6±14	75		0.79
Lamb E J (187), 2003	52	Elderly	80±5	53±18	⁵¹ Cr-EDTA	aMDRD	6±10.2			0.84
Pierrat A (188), 2003	116	Renal transplant	46±13	56	Inulin	MDRD6	-2±12			0.56
Lewis J (189), 2001	1703	African Americans with CKD	54±11	57±24	¹²⁵ Iothalamate	MDRD	-1	90		0.82
Zuo L (190), 2005	261	Chinese with CKD	53±16	68±42	^{99m} Tc-DTPA	aMDRD	3±25	58	74	0.59
Fehrman-Ekholm I (191), 2004	52	Elderly	82	68±11	Iohexol	MDRD	-8			0.53
Froissart M (21), 2005	1044	Routine GFR ≥60 ml/min/1.73m ²		87 ^b	⁵¹ Cr-EDTA	aMDRD	-3±17.2	92	99	
Gonwa T A (192), 2004	1447	Pre liver transplantation	49±11	91±41	¹²⁵ Iothalamate	aMDRD	-7	67	86	0.45
Rule A (20), 2004	580	Kidney donors	41±11	101±17	Iothalamate	aMDRD	-29±24	54		0.19
Poggio E D (174), 2005	457	Kidney donors	42±10	106±18	¹²⁵ Iothalamate	aMDRD	-9	86	98	
Bostom A G (193), 2002	109	CKD + Cr < 1.5 mg/dl	43±13	109 ^b	Iohexol	aMDRD	-42	28	82	0.29
Tello A ^c (194), 2004	1293	Diabetics + Cr < 1.2 mg/dl	13-39 ^a		Iothalamate	aMDRD	-22	78	98	0.13
Lin J (195), 2003	100	Kidney donors	41±10	113±21	¹²⁵ Iothalamate or ^{99m} Tc-DTPA	aMDRD	-18	65	95	0.02
Doorenbos R C ^c (196), 2003	334	Kidney donors + HT	37±14	121±23 ^d	Iothalamate ^d	aMDRD	-31 ^d	61		0.32
Ibrahim H (197), 2005	1286	Type 1 diabetes	34±7	122±23	¹²⁵ Iothalamate	aMDRD	-22	78	98	0.13

Tables 1.3 and 1.4 include all studies of ≥50 individuals which assessed the performance of either an MDRD formula or the Cockcroft-Gault formula compared to a reference GFR method identified by searching MEDLINE 1966-January 2005 using the following search terms: ("Glomerular Filtration Rate" AND ("equation" OR "formula")) OR "MDRD" OR "Cockcroft and Gault" OR "Cockcroft Gault". Studies which included more than 1 measurement per individual or which used measured creatinine clearance or ^{99m}Tc-DTPA renography as the reference method were not included. Data are shown as mean ± standard deviation or percentage unless otherwise indicated.

n = sample size, GFR = glomerular filtration rate measured by reference method, Bias = mean (test method GFR - reference method GFR), SD of the bias = standard deviation (test method GFR - reference method GFR), p30% and p50% = the percentage of individuals in which (|test method-reference method| x 100% / reference method) is ≤30% and ≤50% respectively, r² = the regression statistic indicating the proportion of the variance reference method which is explained by the test method, CKD = chronic kidney disease, MDRD = Modification of Diet in Renal Disease Study equation (116), aMDRD = Abbreviated MDRD equation (114), ⁵¹Cr-EDTA = chromium-51 ethylene diamine tetra-acetate, MDRD6 = MDRD equation 6 (116), ^{99m}Tc-DTPA = ^{99m}Tc-diethylene-triamine-penta-acetic acid, Cr = creatinine, HT = hypertension.

^a range, ^b median, ^c published in abstract only, ^d ml/min (not corrected for body surface area)

Table 1.4: Studies which assessed the performance of the Cockcroft and Gault formula (C&G) compared to a reference method

First author, publication year	n	Population	Age	GFR <i>ml/min/1.73m²</i>	Reference method	Formula	Bias (SD) <i>ml/min/1.73m²</i>	p30%p50% % %	r ²
Poggio E (174), 2004	828	CKD	56±16	32±28	¹²⁵ Iothalamate	C&G	5	60 77	0.79
De Santo N G (124), 1991	62	CKD	49±2	34	Inulin	C&G	0		
Froissart M (21), 2005	1051	Routine GFR <60 ml/min/1.73m ²		34 ^a	⁵¹ Cr-EDTA	C&G	4±9.7	69 85	
Lamb E J (187), 2003	52	Elderly	80±5	53±18	⁵¹ Cr-EDTA	C&G	-5±7.9		0.84
Pierrat A (188), 2003	116	Renal transplant	46±13	56 ^{a,b}	Inulin	C&G ^b	3±15.5 ^b		0.45
Lewis J (189), 2001	1703	African Americans with CKD	54±11	57±24 ^b	¹²⁵ Iothalamate	C&G ^b	-3 ^b	75	0.72
Waller D G (120), 1991	171	Routine GFR		60 ^b	^{99m} Tc-DTPA	C&G ^b	0 ^b		0.87
Itoh K (97), 2003	133	Routine GFR	24-84 ^c	4-122 ^{b,c}	^{99m} Tc-DTPA	C&G ^b	-6±17.4 ^b		0.68
Zuo L (190), 2005	261	Chinese with CKD	53±16	68±42	^{99m} Tc-DTPA	C&G	-8±23 ^b	57 80	0.61
Fehrman-Ekholm I (191), 2004	52	Elderly	82	68±11 ^b	Iohexol	C&G ^b	-22 ^b		0.5
Perlemoine C (198), 2003	89	Routine GFR	61±14	73±41 ^b	⁵¹ Cr-EDTA ^b	C&G ^b	-2 ^b		0.77
Mussap M (199), 2002	52	Type II diabetics	59		⁵¹ Cr-EDTA	C&G ^b	-1 ^b		0.49
Lemann J (200), 1990	136	Diabetics	35±8	78±35 ^b	¹²⁵ Iothalamate ^b	C&G ^b	1±20 ^b		0.67
Hoek F J (201), 2003	123	Routine GFR	50 ^a	78	¹²⁵ Iothalamate	C&G	16±15		0.77
Poole S G (202), 2002	122	Cancer patients	62±12	87±28 ^b	^{99m} Tc-DTPA	C&G ^b	-12 ^b		0.46
Froissart M (21), 2005	1044	Routine GFR ≥60 ml/min/1.73m ²		87 ^a	⁵¹ Cr-EDTA	C&G	0±19.4	88 97	
Gonwa T A (192), 2004	1447	Pre liver transplantation	49±11	91±41 ^b	¹²⁵ Iothalamate	C&G ^b	-9 ^b	61 84	0.42
De Santo N G (124), 1991	62	Healthy volunteers	54±4	103	Inulin	C&G	-5		
Poggio E D (174), 2005	457	Kidney donors	42±10	106±18	¹²⁵ Iothalamate	C&G	3	85 97	
Bostom A (193), 2002	109	CKD + Creatinine <1.5mg/dl	43±13	109 ^{a,b}	Iohexol	C&G ^b	-27 ^b	59 88	0.17
Tello A ^d (194), 2004	1293	Diabetics + Creatinine < 1.2 mg/dl	13-39 ^c		Iothalamate	C&G ^b	-6 ^b	88 97	0.11
Kuhn A ^d (203), 2001	108	Gastroenterology patients	55±12	109±38 ^b	Inulin ^b	C&G ^b	-18 ^b		0.45
Lin J (195), 2003	100	Kidney donors	41±10	113±21	¹²⁵ Iothalamate or ^{99m} Tc-DTPA	C&G	17	58 79	0.06
Lemann J (200), 1990	110	Healthy volunteers	30±8	117±18 ^b	Inulin ^b	C&G ^b	-8±16 ^b		0.41
Doorenbos R C ^d (196), 2003	334	Kidney donors + hypertensives	37±14	121±23 ^b	Iothalamate ^b	C&G ^b	-19 ^b	79	0.38
Ibrahim H (197), 2005	1286	Type 1 diabetes	34±7	122±23	¹²⁵ Iothalamate	C&G	-6	88 97	0.11

The search strategy, inclusion criteria and abbreviations for this table are described in table 1.3. Data are shown as mean ± standard deviation or percentage unless otherwise indicated. ^a median, ^b ml/min (not corrected for body surface area), ^c range, ^d published in abstract only.

Table 1.5: Published formulae to predict GFR from plasma cystatin C concentration in adults

Author, year	Population	Formula
Grubb, 2005 (204)	Routine GFR referrals (n=451)	$\text{GFR (iohexol clearance)} = 83.96 \times [\text{cystatin c}]^{-1.676}$ $\text{GFR (iohexol clearance)} = 86.48 \times [\text{cystatin c}]^{-1.686} \text{ (x 0.948 if female)}$
Hoek, 2003 (201)	Routine GFR referrals (n=93) and patients with type 2 diabetes (n=30)	$\text{GFR (}^{125}\text{-Iothalamate clearance)} = 80.35 / [\text{cystatin c}] - 4.32$
Larsson, 2004 (161)	Routine GFR referrals (n=100)	<p>If Dade PENIA cystatin c method;</p> $\text{GFR (iohexol clearance)}^* = 77.24 \times [\text{cystatin c}]^{-1.2623}$ <p>If Dako PETIA cystatin c method;</p> $\text{GFR (iohexol clearance)}^* = 99.43 \times [\text{cystatin c}]^{-1.5837}$
Le Bricon, 2000 (205)	Renal transplant patients (n=25)	$\text{GFR (}^{51}\text{Cr-EDTA clearance)} = 78 / [\text{cystatin c}] + 4$
MacIsaac, 2006 (206)	Diabetic patients (n=125)	$\text{GFR (}^{99\text{m}}\text{Tc-DTPA clearance)} = 84.6 / [\text{cystatin c}] - 3.2$
Rule, 2006 (162)	Routine GFR referrals; CKD (n=204) Solid organ transplant recipients (n=206)	$\text{GFR (}^{125}\text{-Iothalamate clearance)} = 66.8 \times [\text{cystatin c}]^{-1.30}$ $\text{GFR (}^{125}\text{-Iothalamate clearance)} = 76.6 \times [\text{cystatin c}]^{-1.16}$
Tan, 2002 (207)	Diabetic patients (n=40)	$\text{GFR (iohexol clearance)} = 87.1 / [\text{cystatin c}] - 6.87$

GFR = glomerular filtration rate in ml/min/1.73m² unless otherwise indicated, n = number of individuals in the derivation set, PENIA = particle-enhanced nephelometric immunoassay, PETIA = particle-enhanced turbidometric immunoassay, ^{99m}Tc-DTPA = ^{99m}Tc-diethylene-triamine-penta-acetic acid, ⁵¹Cr-EDTA = chromium-51 ethylene diamine tetra-acetate.

Cystatin c is measured in mg/l.

* ml/min (not adjusted to body surface area)

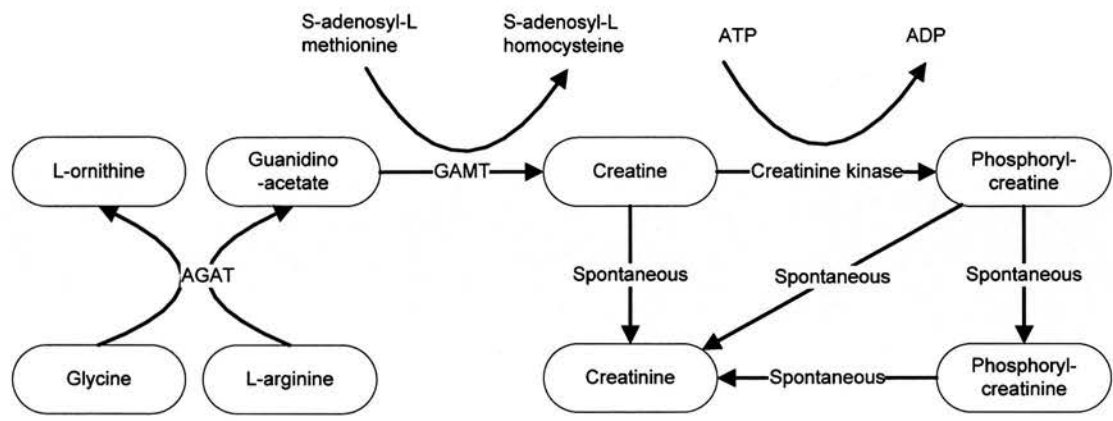
Table 1.6: Studies which assess the performance of formulae to estimate GFR from blood cystatin c concentration compared to a reference method

First author, year	n	Population	Age	GFR <i>ml/min/1.73m²</i>	Reference method	Formula*	Bias (SD) <i>ml/min/1.73m²</i>	p30% %	p50% %
Gerhardt, 2006 (208)	59	Liver transplant recipients	48±12	52.3±17.5	^{99m} Tc-DTPA	Hoek	-0.9±15.9	76	93
						Larsson	-2.8±17.3	70	90
MacIsaac, 2006 (206)	126	Diabetic patients	60	88	^{99m} Tc-DTPA	MacIsaac	-2.2 ±16.4	88	98
Pöge, 2006 (209)	108	Renal transplant patients	49	40	^{99m} Tc-DTPA	Hoek	-0.6 ±9.0	77	97
						Larsson	-3.2 ±9.6	77	95
White, 2005 (210)	117	Renal transplant patients	52±11	58±23	^{99m} Tc-DTPA	Hoek	-10.6 ±11.7	79	
						Larsson	-16.1 ±14.2	53	
						Le Bricon	-3.8 ±11.8	89	

The studies in this table were identified by searching MEDLINE 1966 - 1 April 2007 using the term "Cystatin". Data are shown as mean ± standard deviation or percentage.
n = sample size, GFR = glomerular filtration rate measured by reference method, Bias = mean (test method GFR – reference method GFR), SD of the bias = standard deviation (test method GFR – reference method GFR), p30% and p50% = the percentage of individuals in which (|test method-reference method| x 100% / reference method) is ≤ 30% and ≤ 50% respectively, ^{99m}Tc-DTPA = ^{99m}Tc-diethylene-triamine-penta-acetic acid.

* see table 1.5

Figure 1.1: Generation of creatinine in humans



GAMT, S-adenosyl-L-methionine: N-guanidinoacetate methyl transferase
AGAT, L-arginine:glycine amidinotransferase
ATP, Adenosine triphosphate
ADP, Adenosine diphosphate

Adapted from Wyss et al (101)

Chapter 2: Accuracy of a novel GFR method (blood spot iothexol clearance [BSIC]) and comparison with blood creatinine or cystatin c based equations

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2.1 Study aims

Primary aims of the study;

- To assess the accuracy of a novel method of measuring GFR in which iothexol clearance is measured using 3 dried capillary 'finger-prick' blood samples.
- To determine whether the blood spot iothexol clearance (BSIC) method represents a substantial advantage in terms of test accuracy over creatinine or cystatin c prediction equations, both among those with 'reduced GFR' ($<60 \text{ ml/min/1.73m}^2$) and those with 'preserved GFR' ($\geq 60 \text{ ml/min/1.73m}^2$).

Secondary aims of the study;

- To assess whether the number of sampling points used in the BSIC method can be reduced without loss of accuracy.
- To assess the potential impact of misreporting of sample collection time on test accuracy.
- To determine whether measurement of individual haematocrit is necessary to accurately measure iothexol clearance using dried whole capillary blood samples.

2.2 Methods

2.2.1 *Participant recruitment and data collection*

Sixty consecutive patients referred for assessment of GFR and 21 healthy volunteers were recruited at the South West Thames Institute for Renal Research, St Helier Hospital, Surrey, UK. Participants were instructed to abstain from caffeine, paracetamol, smoking and high protein foods for 6 hours prior to and during the study visit. After giving written informed consent, weight and height were recorded and baseline venous blood samples were taken for measurement of haematocrit, serum creatinine and serum cystatin C concentrations. A bolus of 5ml of 300mg/ml iohexol (Omnipaque® 300, Nycomed Amersham plc, Buckinghamshire, UK) was administered via an intravenous cannula. Venous blood, sampled from the contralateral arm, and capillary blood spots were taken by one of two trained researchers approximately 2, 3 and 4 hours later and the precise times recorded. Capillary blood was drawn using a 'Unistik 2 normal' disposable lancet (Owen Mumford, Oxford, UK). Pressure was applied to the finger to form a large droplet, which was then dropped onto filter paper (Schleicher and Schuell Grade 903, Dassel, Germany) to obtain a spot of at least 7mm in diameter. The study staff were trained to drop only one spot of blood onto each sample area, as layering of blood within a sample can affect the volume of blood contained in a given area of the paper, thereby introducing error. To simulate outpatient conditions, the capillary blood spots were dried and sent to the laboratory through the post. The study was conducted in compliance with the Declaration of Helsinki and approval for the study was obtained from the Local Research Ethics Committee.

2.2.2 Laboratory analysis

Laboratory analysis was conducted at the South West Thames Institute for Renal Research, St Helier Hospital, Surrey, UK. Serum creatinine concentration was measured using a modified kinetic Jaffé reaction (Advia 1650, Bayer, Newbury, Berkshire, UK) and serum cystatin C concentration by a particle-enhanced turbidometric assay (Dako PETIA kit K0071, Denmark) adapted for use on Advia 1650 (Bayer Newbury, Berkshire, UK). For analysis of iohexol concentration in serum, 56µl of the serum sample was treated with 850µl 5% perchloric acid. For analysis of iohexol concentration in dried capillary blood, a 6.3mm punch of the blood spot (assumed to contain 11.2µl of blood) was treated with 170µl 5% perchloric acid and then vortexed for 3 min, ultrasonicated for 15min, incubated for 30 min at room temperature, and spun at 14000g for 10 min (211). Iohexol concentration in these treated samples was then estimated by reverse-phase High Performance Liquid Chromatography (HPLC) (Column – Nucleosil 120; C18 plus 5cm guard column at 30°C; pump- 1.5ml/min; detector wavelength 254nm; range 0.05). The intra- and inter-assay coefficients of variation for the HPLC assay for plasma samples were 2.7% and 6.0% respectively.

Whole blood iohexol concentration in the capillary blood spot was corrected for the venous blood haematocrit to estimate the equivalent serum concentration using the formula

Estimated serum iohexol concentration = whole blood iohexol concentration/ (1-hematocrit).

2.2.3 GFR Calculations

2.2.3.1 Reference method (iohexol clearance from venous samples)

GFR measured by iohexol clearance using 3 serum samples (2, 3 and 4 hours) and corrected for Body Surface Area (BSA; estimated by the Dubois-Dubois formula (95)) was considered the reference method for this study. This was calculated by a one compartment model using the formulae (a)-(c) below (89).

$$(a) \text{ Clearance } (1) \text{ (ml/min)} = \text{Dose (mg/ml)} / (C_0/k)$$

Where C_0 is the intercept and k the slope of the least squares regression line of \ln [iohexol concentration (mg/ml)] against time (minutes).

$$(b) \text{ Clearance } (2) \text{ (ml/min)} = 0.990778 \times \text{Clearance } (1) - 0.001218 \times \text{Clearance } (1)^2$$

$$(c) \text{ GFR (ml/min/1.73m}^2\text{)} = \text{Clearance } (2) \text{ (ml/min)} \times 1.73 / \text{BSA (m}^2\text{)}$$

2.2.3.2 Test methods

(i) Iohexol clearance from capillary blood spots

In the main analysis the 3-spot (2, 3 and 4 hours) and 2-spot (2 and 4 hours) iohexol clearances were calculated using formulae (a)-(c) above. The 1-spot iohexol clearance was calculated from a single capillary blood spot taken at 4 hours using the formulae (d)-(g) below (79). Since it is not possible to estimate the slope of the iohexol elimination curve using blood iohexol concentration taken at a single time point, this formula employs a function of the estimated volume of distribution of iohexol and the dose of iohexol administered as a surrogate for the concentration of iohexol in the blood at time zero.

$$(d) \text{ Clearance }_{(1)} (\text{ml/min}) = \frac{\ln[Dose \text{ iohexol (mg)} / (C_t \times V)]}{0.0016 + t/V}$$

where t = sample time (minutes), C_t = iohexol concentration (mg/ml) at time t , and the volume of distribution (ml), $V = (187 \times \text{weight (kg)}) + 732$ (ml) (212). This estimate of iohexol clearance requires adjustment by a factor which is dependent on the value of the estimate, given as m in the equations below.

$$(e) m = 0.991 - 0.00122 \times \text{Clearance }_{(1)}$$

$$(f) \text{ Clearance }_{(2)} (\text{ml/min}) = \frac{\ln[Dose \text{ iohexol (mg)} / (C_t \times V / m)]}{0.0016 + mt/V}$$

$$(g) \text{ GFR (ml/min/1.73m}^2) = \text{Clearance }_{(2)} (\text{ml/min}) \times 1.73 / \text{BSA (m}^2)$$

In a secondary analysis, the iohexol clearance calculation (formulae (a)-(c) above) was repeated using blood spots taken 2 and 3 hours after the injection, and 3 and 4 hours after the injection.

(ii) Prediction equations

In the main analysis, GFR was estimated from the serum concentration of creatinine ($\mu\text{mol/l}$) and cystatin C (mg/L) using the following equations:

MDRD formula (114)

$$\text{GFR} = 186 \times [\text{Creatinine} / 88.4]^{-1.154} \times [\text{age}]^{-0.203} \times [0.742 \text{ if female}] \times [1.212 \text{ if black}]$$

Cockcroft and Gault formula (115)

$$\text{GFR} = \frac{(140 - \text{age}) \times \text{weight (kg)} \times (1.23 \text{ if male, or } 1.05 \text{ if female})}{\text{Creatinine}} \times 1.73 / \text{BSA (m}^2)$$

Cystatin C formula by Hoek (201)

$$GFR = (80.35 / \text{Cystatin C concentration}) - 4.32$$

For comparison, GFR was calculated GFR from two recently published equations:

The Mayo formula (20)

$$GFR = \exp (1.911 + 5.249 / (\text{creatinine} \times 0.0113) - 2.114 / (\text{creatinine} \times 0.0113)^2 - 0.00686 \times \text{age} - 0.205 \text{ (if female)})$$

The cystatin c equation by Grubb (204)

$$GFR = 86.48 \times \text{cystatin c}^{-1.686} \times 0.948 \text{ (if female)}$$

(iii) Blood spot iohexol clearance methods without haematocrit correction

In order to assess whether adjustment of the whole blood iohexol concentration in the capillary blood spot for the measured venous blood haematocrit is necessary, a haematocrit of 0.39 for women and 0.44 for men (the mean haematocrit in a large community population based survey (30)) was substituted for the measured haematocrit and the 3-spot, 2-spot and 4-hr-spot GFR estimates were re-calculated as detailed above.

2.2.3.3 Statistical methods

Data analysis was undertaken at the Clinical Trial Service Unit, Oxford, UK. The characteristics of routine referral patients and healthy volunteers were compared using 2-sample *t*-tests for normally distributed variables, Wilcoxon rank-sum tests for non-normally distributed variables and Pearson's chi-square test (with appropriate continuity corrections) for dichotomous variables. Agreement of each of the 6 test methods with the reference method was assessed by Bland-Altman plots of the 'GFR difference' (test method GFR – reference method GFR) versus the mean of

the reference method GFR and test method GFR (213). For each method the test 'bias' was defined as the mean of the GFR differences (so that a negative bias corresponds to an underestimation of the GFR by the test method), and the test 'precision' was measured by the standard deviation of the GFR differences (i.e. a smaller standard deviation indicates better precision); 95% limits of agreement were defined as the bias $\pm 1.96 \times$ precision. In order to allow comparisons of the overall performance of each test method (including both components of bias and precision) the mean squared error (MSE) was calculated as the variance of the GFR differences plus the square of the bias. These analyses were repeated for all 3 blood spot iohexol clearance methods calculated using a standard, rather than measured, haematocrit and for the 2 spot and 1 spot methods using alternative sampling times (2 and 3 hours, and 3 and 4 hours respectively).

In a secondary analysis, the performance of each test method in estimating GFR in those with or without chronic kidney disease (CKD) was assessed. This involved estimating the test bias and precision separately in those with a reference GFR <60 ml/min/1.73 m² (the threshold level defined by the National Kidney Foundation for the diagnosis of CKD (16)) and in those with a reference GFR ≥ 60 ml/min/1.73 m². In each group, the 'relative bias' (defined as the mean percentage difference between the test and the reference estimates) was calculated and the differences were compared using a 2-sample t-test.

In order to examine the potential influence on estimated GFR of inaccurate recording of sample time, we performed a sensitivity analysis using simulation. This was done by adding a normally distributed recall error (with mean zero and standard deviation

ranging from 1 to 30 minutes) to the blood spot sample times, recalculating the estimated GFR for each subject, and then recalculating the mean squared error of the test. The above process was repeated 1000 times and, at each level of recall error, the average MSE observed over the 1000 simulations was calculated.

2.3 Results

2.3.1 *Participant characteristics*

The characteristics of the 81 participants are shown in table 2.1. The group of healthy volunteers were younger and had lower median GFR (as estimated by the reference method) than the routine referral patients.

2.3.2 *Performance of the blood spot iohexol clearance methods*

Table 2.2 shows the bias, precision and mean squared error of each of the test methods: the Bland-Altman plots for each test method are shown in figure 2.1. The 3-spot (2, 3 and 4 hours), 2-spot (2 and 4 hours) and 1-spot (4 hours) capillary blood spot iohexol clearance methods measured GFR with small biases, overestimating GFR by only 1.1, 0.6 and 4.5 ml/min/1.73m² respectively. The standard deviation of the GFR differences for the blood spot iohexol clearance methods was similar for the 3-spot and 2-spot methods (7.7 and 7.6 ml/min/1.73m², respectively), and only slightly greater for the 1-spot method (10.8 ml/min/1.73m²). Thus, the 95% limits of agreement for the 3-spot and 2-spot methods were similar, while the limits for the 1-spot method were slightly wider (Table 2.2 and Figure 2.1). The MSE (i.e. the average squared difference between the test estimate and true reference level) of the 1-spot method was approximately twice that of the 2-spot or the 3-spot method (Table 2.2).

2.3.3 *Performance of the creatinine and cystatin c based prediction equations*

The MDRD, Cockcroft-Gault and cystatin c formulae all produced substantially biased estimates of GFR, underestimating GFR by an average of 15.7, 9.6 and 12.1

ml/min/1.73m² respectively (Table 2.2). While the standard deviations of the GFR differences for these methods were similar (17.0, 16.8, and 16.2 ml/min/1.73m² respectively), they were over twice that of the 3-spot method, resulting in much wider 95% limits of agreement. All 3 of these prediction equations resulted in an MSE more than 6 times that of the 3-spot method, with the MDRD formula displaying the worst agreement with the reference method (an MSE nearly 9 times that observed for the 3-spot method). For comparison, 2 recently published prediction equations were also assessed. The 'Grubb' cystatin c formula (204), which includes an adjustment for female sex, also resulted in a biased and imprecise GFR estimate (bias \pm 1.96 x SD: 10.7 \pm 36.3 ml/min/1.73m²). However, the Mayo creatinine equation (20), which includes a quadratic creatinine term, predicted GFR with minimal bias, although the 95% limits of agreement were similar to the other prediction equations (Bias \pm 1.96 SD: 0.3 \pm 30 ml/min/1.73m²).

2.3.4 Performance of test methods among those with and without CKD

While the performance of the 2-spot and 3-spot methods was similar among those with a reference GFR ≥ 60 or < 60 ml/min/1.73m² (Table 2.3), the 1-spot method overestimated GFR by 15.4% among those with GFR < 60 ml/min/1.73m², compared to only 4.8% among those with a reference GFR of > 60 ml/min/1.73m² (p=0.03, Table 2.3). Among those with reference GFR < 60 ml/min/1.73m², the estimates produced by the prediction equations (MDRD, Cockcroft-Gault, and cystatin C formulae) showed little evidence of bias, and the precision was similar to that of the blood spot methods (Table 2.3). However, these equations performed particularly poorly among those with reference GFR ≥ 60 ml/min/1.73m². The bias \pm 1.96 x SD was -25.3 \pm 28.8 for the MDRD formula, -16.8 \pm 32.6 for the Cockcroft-Gault

formula and -19.5 ± 32.0 ml/min/ 1.73m^2 for the cystatin C formula. Among those with a $\text{GFR} \geq 60$ ml/min/ 1.73m^2 the MDRD, Cockcroft and Gault and cystatin C formulae underestimated GFR by an average of 26.4%, 17.5% and 20.0% respectively ($p < 0.005$ for comparison with the relative bias of the same test method among those with a reference $\text{GFR} < 60$ ml/min/ 1.73m^2 , Table 2.3).

2.3.5 Sensitivity analysis: Impact of error in reported sample time

The influence of inaccurate recording of sample time on the expected mean squared error of the 3 spot, 2 spot and 1 spot iohexol clearance test methods is shown in Figure 2.2. All these methods were fairly robust to recall error, unless the standard deviation of the sample time error exceeded 15 minutes. Since the sampling distribution was Gaussian, this is approximately equivalent to the requirement that no more than one third of subjects routinely misreport these times by 15 minutes or more. Under this model, the 3-spot method lost its advantage over the 1-spot method once the SD of the sample time error exceeded 30 minutes. This is because the 1-spot method assumes a common gradient between dose and time and is thus less sensitive to errors in the recording of the time of the blood sample.

2.3.6 Sensitivity analysis: Impact of blood haematocrit measurement

When a standard haematocrit value (0.44 for men and 0.39 for women) was used in place of the measured haematocrit, the 2-spot and 3-spot methods produced accurate GFR estimates (Bias \pm 1.96 SD: 0.2 ± 15.7 ml/min/ 1.73m^2 and 0.7 ± 15.9 ml/min/ 1.73m^2 respectively, Table 2.4). However, for the 1-spot method, the use of a standard haematocrit value resulted in wide 95% limits of the agreement, and

consequently large MSE (Bias \pm 1.96 SD: 4.6 ± 32 ml/min/1.73m², MSE 290.7, Table 2.4).

2.3.7 Sensitivity analysis: Impact of sampling time

The 1-spot method reported in the main analysis was based on a single blood spot collected at 4 hours. The GFR estimates produced by the 1-spot method were less accurate when a single blood spot collected at 2 or 3 hours was used (Bias \pm 1.96 SD: 7.5 ± 30.6 ml/min/1.73m² and 7.4 ± 20 ml/min/1.74m² respectively, Table 2.5).

Similarly, the 2-spot methods using blood spot samples taken at 2 and 3 hours, or 3 and 4 hours were substantially less precise (Bias \pm 1.96 SD: -1.6 ± 38 and -4.9 ± 36.1 ml/min/1.73m² respectively, table 2.5) than 2-spot method using blood sampled at 2 and 4 hours reported in the main analysis (Bias \pm 1.96 SD: 0.6 ± 14.9).

2.4 Discussion

This study has demonstrated that, as compared to iohexol clearance estimated from venous samples taken at 2, 3 and 4 hours after iohexol injection, a novel method of measurement of iohexol clearance using timed dried capillary blood spots can estimate GFR with low bias and clinically acceptable precision. This study suggests that 3-spot method provides little statistical advantage over the 2-spot method, but that the 1-spot method, although it measures GFR with low bias, may result in some loss of precision.

This new method has two key potential advantages for large scale epidemiological studies or trials compared to traditional iohexol clearance measured using timed venous blood samples, or other exogenous tracer based methods of estimating GFR. First, since iohexol in blood spots appears to be stable when sent through the mail without refrigeration (211), GFR could be estimated in a multi-centre study without the need for calibration of local laboratories or expensive transfer of venous samples to a central laboratory. Second, following the iohexol injection, this method could potentially be completed by the subject at home, which would reduce the time that study participants would need to spend in clinics.

2.4.1 *Performance of the creatinine and cystatin c based prediction equations*

In this study, the creatinine-based (MDRD (114) and Cockcroft-Gault (115)) and cystatin-C-based (201) prediction equations performed poorly among individuals without CKD (i.e. those with $\text{GFR} \geq 60 \text{ ml/min/1.73m}^2$). However, among patients with established CKD ($\text{GFR} < 60 \text{ ml/min/1.73m}^2$) the performance of these equations was comparable to the more direct methods. This suggests that the chief

advantage of the blood spot method is likely to be in clinical (and especially epidemiological) studies requiring accurate measurement of GFR among individuals without known CKD, in whom prediction equations are inadequate (20;21;174).

In this sample, both the MDRD and the Cockcroft-Gault equations substantially underestimated the reference GFR among those without CKD. When GFR is estimated from serum creatinine concentration, the bias of the estimate is critically dependent on the calibration of the creatinine assay (167). Recently, the MDRD formula has been re-expressed for use with creatinine assays which are standardized to an isotope dilution mass spectrometry (IDMS) reference method (214) (section 1.3.1.3). In this study, we were unable to calibrate the creatinine assay, either to the original MDRD laboratory method, or to an IDMS reference method. Therefore, the extent to which the underestimation of GFR by the MDRD equation in this study is the result of differences in creatinine assay calibration is unknown. We therefore compared our results with those from published studies assessing the performance of an MDRD or the Cockcroft-Gault formula. In figure 2.3, the mean or median reference GFR for each of the studies listed in tables 1.3 and 1.4 is plotted against the reported bias (test method – reference method). There is a tendency for these formulae, in particular the MDRD formulae, to systematically underestimate GFR in populations with higher GFR levels. Using measurement of ¹²⁵I-iothalamate clearance in 580 healthy potential kidney donors and 320 patients with CKD at the Mayo clinic in the US, Rule et al found that for a given blood creatinine concentration, GFR was on average 26% higher in healthy individuals than in patients with chronic kidney disease of the same age and sex (20). That study developed a new prediction equation which included a quadratic creatinine term to

account for this non-linear relationship between GFR and reciprocal creatinine observed when the full population GFR range is considered (Table 1.2) (20). In our data, this new 'Mayo' equation resulted in an unbiased estimate of GFR; however the imprecision of this formula was similar to the MDRD or Cockcroft-Gault formulae.

2.4.2 Performance of blood spot iohexol clearance using earlier time points and without measurement of blood haematocrit

This study was able to further explore how blood spot iohexol clearance could be used in a large scale epidemiological study by examining whether earlier time points could be used without loss of accuracy, and whether measurement of haematocrit, which would require either local measurement or refrigerated transport of blood samples to the research laboratory, is necessary.

First, in the main analysis, the 2-spot method used samples taken at 2 and 4 hours while the 1-spot method used only the 4 hour sample. The use of earlier time points (2 and 3 hours for the 2-spot method, and 2 hours for the 1-spot method) might be more convenient for participants and research staff. However, both the 1-spot and 2-spot methods were substantially less accurate when these earlier time points were used (Table 2.5).

Second, substituting the mean haematocrit values for men and women from a population survey (30) in place of measured haematocrit did not affect the performance of the 2-spot or 3-spot iohexol method, but led to a loss of precision in the 1-spot method.

2.4.3 Limitations of the study

First, the blood spot sampling was performed by study staff. For large scale studies, one of the key advantages of this method compared to existing exogenous tracer based GFR methods is the potential for the blood spots to be collected by the participants themselves at home. However, further research is needed to establish whether subjects would themselves be able to take adequate blood spot samples after a short training within a study clinic appointment.

Second, the precise sample time was recorded by the researcher in this study. If the samples were collected by participants at home, misreporting of the blood spot sample collection could affect test accuracy. We were able to simulate the potential impact of misreporting of sample time on test performance by adding a randomly distributed error variable to the recorded time in our data. This showed that, even if a third of patients misreported the times with an error of 15 minutes, there would be little reduction of the accuracy of the 3 spot, 2 spot or 1 spot methods (Figure 2.2).

Third, estimation of GFR from the clearance of iohexol from the blood measured using 3 timed venous samples was used as the reference method in this study rather than inulin or ^{51}Cr -EDTA clearance. The choice of reference method was pragmatic and the clearance of a single bolus of iohexol has been shown in previous studies to be equivalent to the gold standard methods of inulin (90;215) and ^{51}Cr -EDTA clearance (89;178).

Fourth, because of the reference method used, this study is not able to reliably assess whether the blood spot iohexol clearance method, or the formulae estimating GFR from blood concentration of creatinine or cystatin c, can accurately estimate true

GFR among individuals with stage 4 or 5 CKD ($\text{GFR} < 30 \text{ ml/min/1.73m}^2$ (16)).

Among such individuals, plasma iohexol clearance measured using a late plasma sample, usually taken around 24 hours after the iohexol injection, better reflects renal iohexol clearance than the reference method used in this study (216). Modification of the blood spot method to include a late sampling point and further validation would be required before using this method among individuals with stage 4 or 5 CKD.

2.5 Summary of key findings

- Blood spot iothexol clearance measured using 3 dried capillary blood spot samples taken at 2, 3 and 4 hours following an iothexol injection accurately measured GFR among individuals with a wide range of GFR values.
- Using 2 blood spots sampled at 2 and 4 hours resulted in similar accuracy to the 3 spot method.
- Using a single blood spot collected at 4 hours resulted in some loss of precision and, among those with $\text{GFR} < 60 \text{ ml/min/1.73m}^2$, led to an overestimation of GFR.
- Among those with $\text{GFR} > 60 \text{ ml/min/1.73m}^2$ the MDRD, Cockcroft-Gault and cystatin C formulae substantially underestimate GFR and produce an imprecise estimate for an individual.
- The 1 spot method using earlier sampling times (2 or 3 hours rather than 4 hours) resulted in greater bias and less precision.
- The 2-spot method was substantially less accurate when the sampling times were close together (2 and 3, or 3 and 4 hours, rather than 2 and 4 hours).
- Measurement of individual blood haematocrit values is not necessary when the 2- or 3-spot methods are used.
- The accuracy of all 3 blood spot methods was not substantially affected by a simulated scenario in which the sampling time was misreported by more than 15 minutes in approximately 1/3rd of participants.
- The blood spot iothexol method has potential for use in large scale epidemiological studies.

Chapter 2

Tables and figures

Table 2.1: Participant characteristics

Characteristic	Routine referrals n=60	Volunteers n=21	All participants n=81	p
Age (years) ^a	58.2 (16.0)	40.0 (11.9)	53.5 (17.0)	<0.001 *
No of women, n (%)	26 (43.3%)	13 (61.9%)	39 (48.1%)	0.225 †
Height (cms) ^a	167.8 (9.7)	168.8 (9.3)	168.1 (9.5)	0.690 *
Weight (kgs) ^a	75.6 (14.1)	73.1 (12.4)	75.0 (13.7)	0.447 *
Serum creatinine (μmol/l) ^b	126.4 (99.0-174.1)	90.2 (85.7-99.9)	106.1 (88.4-157.4)	<0.001 ††
Serum cystatin C (mg/l) ^b	1.54 (1.23-2.12)	0.89 (0.86-0.94)	1.29 (0.96-1.84)	<0.001 ††
Haematocrit (%) ^a	38.5 (5.1)	42.4 (4.1)	39.5 (5.1)	<0.001 *

Reference GFR (ml/min/1.73m ²) ^b	51.9 (34.0-83.0)	100.5 (95.2-104.9)	71.7 (40.1-99.3)	<0.001	†
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^a Continuous variables with an approximately normal distribution are expressed as mean (standard deviation)

^b Continuous variables which are not normally distributed are expressed as median (interquartile range)

P values relate to the comparison between routine referrals and volunteers based on: * a 2 sample t-test; † Pearson chi square test; and ‡ the Wilcoxon rank-sum test. To convert serum creatinine concentration in µmol/l to mg/dl, divide by 88.4.

Table 2.2: Agreement of each test method with the reference method of iohexol clearance measured in three venous samples.

Test method	Bias	SD	Limits of agreement	MSE
Blood spot methods:				
3 spot iohexol clearance	1.1	7.7	-13.9 to 16.2	60.0
2 spot iohexol clearance	0.6	7.6	-14.3 to 15.5	58.2
1 spot iohexol clearance	4.5	10.8	-16.8 to 25.7	137.3
Prediction equations:				
MDRD formula	-15.7	17.0	-49.1 to 17.6	536.6
C&G formula	-9.6	16.8	-42.5 to 23.2	374.1
Cystatin C formula	-12.1	16.2	-43.7 to 19.6	406.7

Values are given in ml/min/1.73m²

Bias = Mean (test method GFR - reference method GFR), SD = Standard deviation (test method GFR - reference method GFR), Limits of agreement = Bias±1.96 x SD, MSE = Mean squared error, MDRD = Modification of Diet in Renal Disease study (114), C&G = Cockcroft and Gault (115)

Table 2.3: Agreement of each test method for estimation of GFR with the reference method among individuals with GFR <60 ml/min/1.73m² and GFR ≥60 ml/min/1.73m².

Test method	GFR <60 ml/min/1.73m ² <i>n</i> = 33	GFR ≥60 ml/min/1.73m ² <i>n</i> = 48	<i>p</i> *
	Mean GFR = 36.2 ml/min/1.73m ²	Mean GFR = 93.0 ml/min/1.73m ²	
	Bias±1.96xSD ml/min/1.73m ²	Bias±1.96xSD ml/min/1.73m ²	Relative Bias
Blood spot methods:			
3 spot iohexol clearance	0.7±14.1	1.4±15.8	1.4%
2 spot iohexol clearance	0.8±14.5	0.5±15.3	0.4%
1 spot iohexol clearance	5.4±16.6	3.8±24.0	4.8 %
Prediction equations:			
MDRD formula (114)	-1.9±16.5	-25.3±28.8	-26.4%
C&G formula (115)	0.7±20.4	-16.8±32.6	-17.5%
Cystatin C formula (201)	-1.2±14.9	-19.5±32.0	-20.0%

Bias = Mean (test method GFR - reference method GFR), SD = standard deviation (test method GFR - reference method GFR), Relative Bias represents the mean of the relative GFR differences (where the GFR difference is expressed as a proportion of the reference GFR), MDRD = Modification of Diet in Renal Disease study, C&G = Cockcroft and Gault

*relative bias when GFR ≥60 ml/min/1.73m² compared with relative bias when GFR <60 ml/min/1.73m²

Table 2.4: Agreement of the Blood Spot Iohexol Clearance (BSIC), without measured haematocrit, with the reference method of iohexol clearance measured in three venous samples.

Blood spot methods	Bias	SD	Limits of agreement	MSE
3 spot iohexol clearance	0.7	8.1	-15.2 to 16.5	65.8
2 spot iohexol clearance	0.2	8.0	-15.5 to 15.8	63.8
1 spot iohexol clearance	4.6	16.4	-27.5 to 36.8	290.7

Bias = Mean (test method GFR - reference method GFR), SD = Standard Deviation (test method GFR - reference method GFR), Limits of agreement = Bias±1.96 x SD, MSE = Mean Squared Error

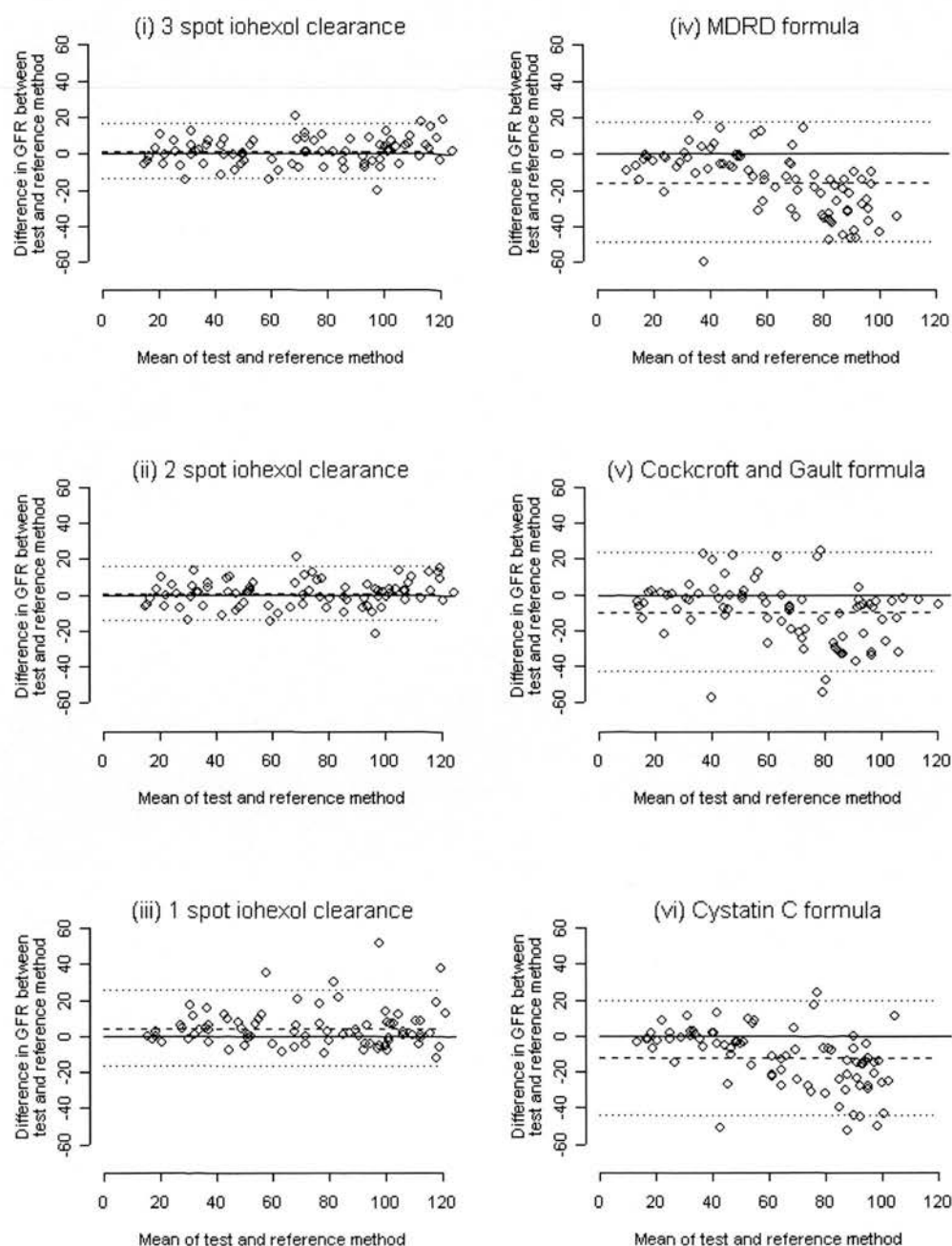
Table 2.5: Agreement of Blood Spot Iohexol Clearance (BSIC), measured using alternative sampling times, with the reference method of iohexol clearance measured using 3 venous samples

BSIC methods	Bias	SD	Limits of agreement	MSE
1-Spot method using different sampling times:				
2 hour	7.5	15.6	-23.2 to 38.1	299.7
3 hour	7.4	10.2	-12.6 to 27.4	171.4
4 hour (as used in main analysis)	4.5	10.8	-16.8 to 25.7	137.3
2-Spot method using different sampling times:				
2 and 3 hours	-1.6	19.3	-36.3 to 36.3	376.3
3 and 4 hours	-4.9	18.4	-40.9 to 31.0	360.9
2 and 4 hours (as used in main analysis)	0.6	7.6	-14.3 to 15.5	58.2

Values are given in ml/min/1.73m²

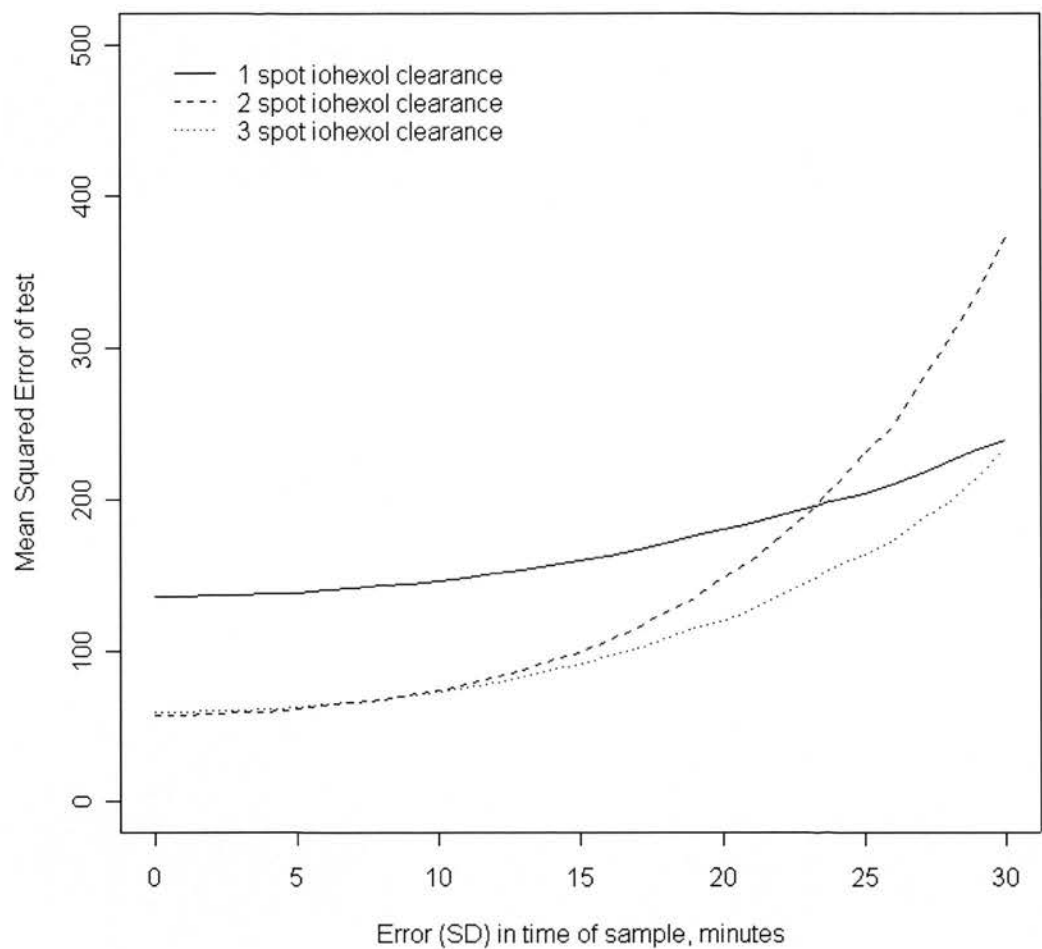
Bias = Mean (test method GFR - reference method GFR), SD = Standard Deviation (test method GFR - reference method GFR), Limits of agreement = Bias±1.96 x SD, MSE = Mean Squared Error

Figure 2.1: Bland and Altman plots showing agreement of (i) 3-spot iohexol clearance, (ii) 2-spot iohexol clearance, (iii) 1-spot iohexol clearance, (iv) the MDRD formula (114), (v) the Cockcroft-Gault formula (115) and (vi) the Cystatin C formula (201) with the reference method of iohexol clearance measured in three venous samples.



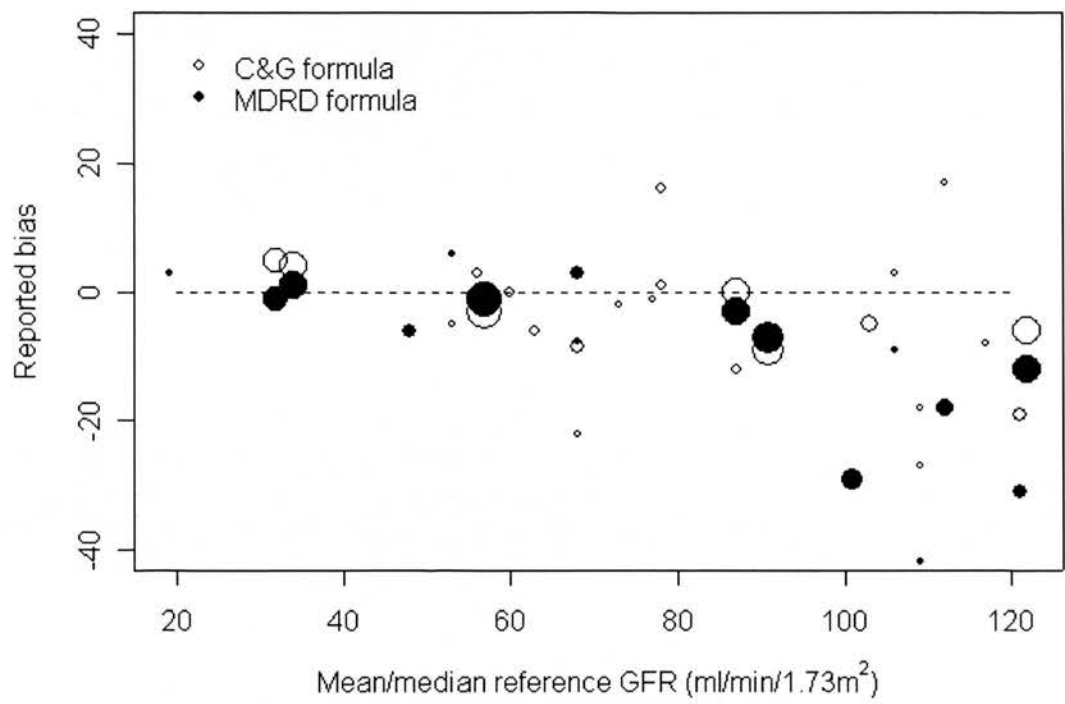
For each individual the 'GFR difference' (test method GFR - reference method GFR) on the y axis is plotted against the 'mean GFR' (mean of the reference and test methods). The bias (calculated as the mean GFR difference) is shown by the dashed line. The upper and lower 95% limits of the agreement [$\text{bias} \pm 1.96 \times \text{SD}$] are indicated by the upper and lower dotted lines.

Figure 2.2: The effect of adding of random error to the recorded blood spot sample time on the accuracy of the 3 spot, 2 spot and 1 spot iohexol clearance methods.



To simulate the effect of error in recording sample time, random error from a normal distribution with a mean of zero minutes and a Standard Deviation (SD) of x minutes (with x allowed to vary from 0-30 minutes) was added to each of the 3 recorded blood spot sample times for all 81 participants and GFR recalculated using the 3 spot, 2 spot and 1 spot test methods for each individual. The Mean Squared Error (MSE) of each recalculated test method was then calculated for the study population. This process was repeated 1000 times and the mean MSE over the 1000 repetitions was calculated.

Figure 2.3: Reported bias in studies assessing the performance of the Modification of Diet in Renal Disease or the Cockcroft Gault formula compared to a reference method



For each of the studies listed in table 1.3 and 1.4 the reported bias [mean (test method GFR – reference Method GFR)] of the Cockcroft and Gault (115) and/or an MDRD formulae (114;116), are plotted against the mean or median reference GFR of the study population. One study (194), published only in abstract, is not included as it did not provide the reference GFR of the study population.

Chapter 3: Feasibility of a new GFR method for use in epidemiological studies

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3.1 Aims of the study

In Chapter 2, GFR measured by a novel method in which iothexol clearance is assessed using dried capillary blood samples (blood spot iothexol clearance or 'BSIC') showed good agreement with traditional iothexol clearance measured using 3 timed venous blood samples. However, in that study, and in other published studies which have used dried capillary blood spot samples (217-219), the blood spot samples were collected by trained staff. One of the principal advantages of this method for epidemiological studies, however, is the potential for the blood sample collection to be undertaken by the participants at home after the clinic.

This study aimed to assess the feasibility, including acceptability to patients, of using the BSIC method, in which the blood sampling is completed by participants themselves after the clinic. In particular, the study aimed to address the following methodological issues;

- the feasibility of teaching participants to take capillary blood spot samples within a single clinic visit,
- the ability of participants to collect and return 2 timed blood spot samples to the clinic, and to record the sample collection time, and
- the size and quality of samples returned by participants.

The study was conducted in compliance with the Declaration of Helsinki and approval for the study was obtained from the Local Research Ethics Committee.

3.2 Methods

3.2.1 *Recruitment of participants*

This study aimed to recruit a wide range of individuals to enable the assessment of the feasibility of the blood spot iohexol method in men and women, across a wide range of age groups, and among healthy individuals as well as those with prior disease.

Participants were recruited from the following sources;

- Relatives of outpatients followed up at the Oxford Kidney Unit,
- Participants in the Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) and their relatives,
- Clinical Trial Service Unit staff and other members of the University of Oxford

Details of the recruitment methods for the different participant sources are described below:

3.2.1.1 Relatives of outpatients followed up at the Oxford Kidney Unit

A few weeks before their next scheduled outpatient appointment, patients attending the Oxford Kidney Unit outpatient department were sent a letter asking them give an invitation letter and participant information sheet to any family member or friend who be accompanying them to their outpatient appointment, and complete and return a reply slip. On the reply slip the Oxford Kidney Unit patient was asked to indicate one of the following responses;

- “My family member is interested in taking part in this study. Please contact them at the address below to arrange an appointment”
- “I attend the Oxford Kidney Unit outpatient department with a member of my family but they are not interested in taking part in this study”
- “This invitation is not relevant to me because I attend the Oxford Kidney Outpatient Department without a member of my family”

3.2.1.2 Participants in the SEARCH study and their relatives

Approximately 260 of the 12,000 SEARCH participants were enrolled at the Oxford site. By December 2004, around 240 individuals were attending 6 monthly follow-up at the Clinical Trial Service Unit. A few weeks before their next scheduled follow-up appointment, these participants were sent a letter inviting them, and/or a family member or friend who would be accompanying them to the clinic, to participate in this study on the same day as their SEARCH appointment. The SEARCH participant was asked to complete a reply slip indicating one of the following responses;

- “I am interested in taking part in this study. Please contact me to arrange an appointment”
- “I am not interested in taking part in this study”

3.2.1.3 Clinical Trial Service Unit staff and other members of the University of Oxford

Approximately 180 individuals work within the Clinical Trial Service Unit.

Approximately a further 150 individuals work in other University Departments on the same site (the National Perinatal Epidemiology Unit, the Cancer Epidemiology Unit and

the Division of Public Health and Primary Health Care). An invitation letter was sent to every member of staff in the 4 units requesting volunteers for this study.

3.2.2 Eligibility

Inclusion criteria;

Men and women aged ≥ 18 years

Exclusion criteria;

- History of chronic kidney disease requiring renal replacement therapy
- History of multiple myeloma, sickle cell disease or uncontrolled thyroid disease
- Acute illness requiring hospital admission within the last month
- Known hypersensitivity to radiographic contrast media
- Childbearing potential
- Life-threatening illness (e.g. cancer)

Myeloma, sickle cell disease and uncontrolled thyroid disease are relative contraindications to the use of iohexol (93) and the safety of iohexol has not been established in pregnancy.

3.2.3 Clinic visit

Participants attended a single clinic which included the following;

- Assessment of eligibility
- Informed consent
- Medical history

- Clinical measurements
- Laboratory sample collection including the collection of dried capillary blood spots after the clinic

Clinic study visits were conducted by trained personnel (Research Fellow [MM] or Study Research Nurse) and data were collected using a standard case report form. Each participant was allocated a unique 4 digit study ID.

3.2.3.1 Assessment of eligibility

Identifying data (Title, Name, Date of Birth, Sex, Address, General Practitioner, unique study ID) and recruitment source (University staff, SEARCH study, Oxford Kidney Unit, Other) were recorded. Each exclusion criteria was discussed with the participant.

Childbearing potential (women in whom current pregnancy was a possibility) was determined by the participants themselves. Participants with a permanent pacemaker in situ could take part in the study but did not have the bioimpedance measurement.

3.2.3.2 Informed consent

Following a full explanation of the study, patients were asked to provide written informed consent for the study procedures. In addition, participants provided consent for the long term storage of blood and urine sample for use in future medical research.

3.2.3.3 Medical history

Study staff specifically asked about the following conditions;

- (i) Chronic Kidney Disease

Chronic Kidney Disease was considered to be present if the patient reported a history of “kidney damage” or “kidney disease”.

(ii) Angina (stable or unstable)

Angina was considered to be present if the participant reported a diagnosis of angina, a history of exertional chest pain or had been admitted to hospital with chest pain without other cause.

(iii) Myocardial infarction (MI)

A history of MI was self reported by participants.

(iv) Coronary revascularisation

A history of coronary revascularisation was considered present if the participant reported a history of coronary artery bypass surgery or coronary angioplasty with or without stenting.

(v) Non-coronary revascularisation

A history of non-coronary revascularisation was considered to be present if the participant reported a history of non-coronary angioplasty or arterial revascularisation surgery (carotid endarterectomy, abdominal aneurysm repair or peripheral arterial bypass surgery)

(vi) Peripheral Vascular disease (PVD)

A history of PVD was considered to be present if the participant reported a history of intermittent claudication.

(vii) Stroke

A history of stroke was considered present if the participant reported a physician diagnosis of stroke or a history of sudden onset neurological deficit lasting > 24 hours.

(viii) Diabetes Mellitus

A history of diabetes mellitus was considered to be present if the participant was receiving treatment for diabetes or reported a physician diagnosis of diabetes.

(ix) Treated hypertension

A history of treated hypertension was considered to be present if participants reported taking antihypertensive treatment without other indication.

(x) Smoking status

Participants were recorded as current smokers (if they continued to smoke or had stopped smoking in the last month) or ex-smokers.

3.2.3.4 Ethnic origin

Participants were asked to classify themselves as one of the following;

- White
- Black / Afro-Caribbean
- Indian sub-continent
- Oriental
- Other

3.2.3.5 Current medication

A complete list of the participant's current medication (without dose information) was recorded.

3.2.3.6 Clinical measurements

After removing footwear, height was measured to the nearest 0.5 cm using a wall mounted chart. Weight was measured to the nearest 0.1cm using a Tanita Body Composition Analyser (TBF-300, TANITA UK Ltd, Middlesex, UK) after removing shoes and heavy garments. After removal of outer clothing the natural waist was measured to the nearest cm. Blood pressure was measured once while seated using a British Hypertension Society approved digital sphygmomanometer (Model UA-767, A&D Instruments Ltd, Oxfordshire, UK). Provided the participant does not have permanent pacemaker in, an assessment of body composition was made by measurement of single frequency bioimpedance using a Tanita Body Composition Analyser (TBF-300, TANITA UK Ltd, Middlesex, UK). After voiding, outer garments, pocket contents, metal jewellery, shoes and socks were removed. The participant was then asked to step onto the analyser with one foot on each foot plate and a single measurement was made in which the analyser passes a 50Hz current through the lower limbs. The TANITA TBF-300 analyser reports the following parameters;

- measured impedance (in ohms),
- total body water (calculated according to the manufacturers prediction equations from weight, age and $\text{height}^2/\text{impedance}$),

- fat free mass (FFM) (based on the manufacturers assumption that 73.2% of FFM is water), and
- percentage body fat ($[(\text{weight}-\text{FFM})/\text{weight}]$).

3.2.3.7 Blood spot blood sample collection and administration of iohexol

The participant was trained and observed taking 'finger prick' capillary blood spots using a 'Unistik 2 normal' disposable lancet (Owen Mumford, Oxford, UK), applying pressure to the proximal finger and dropping two capillary blood spots onto the blood collection card (Schleicher and Schuell Grade 903, Dassel, Germany) from a single 'finger prick'. The blood spots had to be at least 7mm in diameter. The participant was instructed not to 'smudge' the spot, by touching the card with the finger, or to 'layer' the spot, by dropping several spots on the same area of the card. If sufficient spots could not be obtained, then a 'Unistik 2 super' disposable lancet (Owen Mumford, Oxford, UK) was used.

An intravenous cannula was then inserted, venous blood samples drawn (9ml EDTA and 5ml Sodium Fluoride EDTA) and an intravenous bolus of 5ml (300mg/ml) iohexol was administered. The participant was then given a blood spot sampling kit (which includes written instructions, a blood spot card labelled with their study ID, three disposable lancets, cotton wool, 2 re-sealable plastic bags and a re-sealable SHARPS disposal container). The participant was asked to undertake further capillary blood sampling at approximately 2 and 4 hours after the iohexol injection. From each 'finger prick' two capillary blood spots were to be collected and the precise time (to the nearest minute)

recorded on blood spot card. Participants were instructed to allow the blood spot samples to dry for 30 minutes before placing each completed blood spot card in a sealed plastic bag. The participant was asked to return the dried capillary blood spots to the clinic, along with a completed questionnaire, by post using the envelope provided. The participant was observed for 15 minutes after the administration of iohexol before leaving the clinic.

3.2.3.8 Participant questionnaire

After completing the blood spot sample collection after the clinic, participants filled in a short questionnaire in which they selected the most appropriate answer to the following questions:

(i) I found the iohexol injection at the clinic to be:

Very acceptable

Acceptable

Neutral feeling

Unacceptable

Very unacceptable

(ii) Overall, I found taking the finger prick blood spots to be:

Very acceptable

Acceptable

Neutral feeling

Unacceptable

Very unacceptable

(iii) In my experience, actually pricking the finger using the needle was:

Very easy

Easy

Neutral

Difficult

Very difficult

(iv) I found dropping the blood spots onto the filter paper to be:

Very easy

Easy

Neutral

Difficult

Very difficult

The questionnaire was then returned to the clinic along with the dried capillary blood spots.

3.2.3.9 Sample storage and laboratory methods

Venous blood samples were stored at -4°C until transfer to the laboratory. Whole blood removed from the EDTA blood sample was analysed for haematocrit and haemoglobin (GEN-S, Beckman Coulter, Bucks, UK). Eighty eight percent of samples were processed on the same day as collection with the remaining samples processed the following day.

Completed blood spot collection cards were returned to the study clinic by first class post. A photocopy of each card was made in order to store the recorded the blood spot sample time and provide a record of sample quality. The samples were the stored at - 80°C. The blood spot samples were then transferred in batches to the South West Thames Institute of Renal Research by courier at room temperature. The methods used to analyse blood iohexol concentration in dried capillary blood spots, and the calculations used to estimate GFR corrected for body surface area from 2 blood spots are outlined in section 2.2.3.1. For comparison, GFR was also estimated from blood creatinine concentration by the abbreviated MDRD formula (114) and from blood cystatin c concentration according to the published formula by Hoek et al (201) described in section 2.2.3.2

3.2.3.10 Data entry and storage

Data collected in the clinic, the results of the questionnaire and the blood spot collection times recorded by the participants were manually entered onto a spreadsheet (Microsoft Excel® 2002) by one investigator [MM]. Each row of data was checked by the same investigator on a separate day.

The concentration of iohexol in the blood spots was analysed in the South West Thames Institute of Renal Research, St Helier Hospital and the results were recorded on a separate spread sheet (Microsoft Excel® 2002). The quality of the blood spots was subjectively assessed by a Consultant Biochemist at the South West Thames Institute of Renal Research, St Helier Hospital (JB). Spots which were too small to allow

quantification of the iohexol concentration were considered 'inadequate'. Blood spots which were analysable for iohexol concentration but were either small, or showed 'multiple spotting' (several blood spots applied to the same area) or 'smudging' (direct contact between the filter paper and the finger) were considered 'poor quality' (Figure 3.1). The results of the laboratory tests measured in the venous baseline blood sample were exported from the Clinical Trial Service Unit Laboratory Information Management System (LIMS) as a spreadsheet (Microsoft Excel® 2002). Data was stored in central Clinical Trial Service Unit server.

3.2.4 Statistical methods

Data were expressed as mean \pm standard deviation and range for continuous data and as percentage for categorical data. The results of the study questionnaire were displayed as a pie chart (Microsoft Excel® 2002). Possible 'rounding up' of reported sampling time was explored by analysis of digit preference. The proportion of recorded times in which the last digit was "0" was compared with the expected frequency of 10% using a chi squared test (Microsoft Excel® 2002) (220). The distributions of GFR measured by blood spot iohexol clearance (BSIC-GFR) and GFR estimated from blood concentrations of creatinine (MDRD-GFR) and cystatin c (cyc-GFR) (restricted to individuals who returned 2 analysable blood spot iohexol samples and had measurements of blood creatinine and cystatin c concentrations (n=106)) were displayed as box and whisker plots showing the median, interquartile and 95% range (Stata v8.2 (StataCorp, Texas, USA)). Participants were considered to have a 'high GFR' if the BSIC-GFR value was above 133 ml/min/1.73m² (the 95th centile of measured iohexol

clearance among 365 healthy potential donors (173)) and were considered to have a 'low GFR' if the BSIC-GFR value was below 70 ml/min/1.73m² (the 5th centile of GFR from the same population (173)). The characteristics of those with a 'high GFR' and a 'low GFR' were compared to those with a 'normal GFR' using a chi squared test for categorical variables and a 2 sample t-test for continuous variables (Stata v8.2 (StataCorp, Texas, USA)).

3.3 Results

3.3.1 Participant recruitment and characteristics

Of the 111 eligible participants, 53 were recruited through the SEARCH clinic, 43 from University Staff and 15 through the Oxford Kidney Unit. Around 28% of invited SEARCH participants took part (Figure 3.2). Approximately 13% of staff at the Clinical Trial Service Unit, National Perinatal Epidemiology Unit, Cancer Epidemiology Unit and Department of Public Health and Primary Care volunteered for the study. Around 9% of invitations sent to Oxford Kidney Unit patients resulted in a family member volunteering for the study (Figure 3.2) although the number of Oxford Kidney Unit patients who attended the outpatient department with a family member is not known. The characteristics of the 111 eligible participants are shown in table 3.1.

3.3.2 Quality of returned finger-prick blood samples

All 111 participants successfully collected baseline finger-prick blood samples in the clinic. One hundred and eight participants (97.3%) returned blood spot samples from both time points. Two further participants returned only a single sample (Figure 3.3).

Of the 108 participants who returned 2 samples, only 1 returned a sample which was too small to allow quantification of the iohexol concentration. However, 24 participants returned samples which, while they were analysable, were either small or had been collected using 'poor' technique, e.g. 'smudging' (touching the blood spot collection

card with the finger) or ‘multiple spotting’ (the application of several spots to the sample area of card) (Figure 3.1).

3.3.3 Recording of sample time by participants

The sample time was recorded clearly and legibly on all 218 returned blood sample cards. Among the 108 participants who returned samples from both time points, the recorded sample times for the first (2 hour) sample ranged from 108 minutes to 192 minutes after the iohexol injection, with a mean (\pm standard deviation) recorded time interval of 125 ± 10 minutes. The recorded sample times for the second (4 hour) sample ranged from 193 minutes to 318 minutes after the iohexol injection, with a mean (\pm standard deviation) recorded time interval of 245 ± 14 minutes. Among those who returned 2 samples, 94% of the recorded 2 hour sample times and 91% of the recorded 4 hour sample times were within 15 minutes of the scheduled sampling time. In all 218 returned samples, examination of the frequency of the last digit used in recording the sample time demonstrated a preference for the digit “0” which was recorded as the last digit in 22% of time records compared to the expected 10% (Chi^2 25.1, $p<0.0001$) (Figure 3.4).

3.3.4 Acceptability questionnaire results

One hundred and eight participants returned a completed acceptability questionnaire. Ninety four percent of those who returned the questionnaire found the iohexol injection in the clinic to be either “acceptable” or “very acceptable” (Figure 3.5a) and 90% of responders found taking the blood sample after the clinic using the finger-prick method

to be either “acceptable” or “very acceptable” (Figure 3.5b). When asked in detail, 90% of respondents found actually pricking the finger “easy” or “very easy” (Figure 3.6a) but only 72% found dropping the blood spot onto the filter paper “easy” or “very easy” (Figure 3.6b).

3.3.5 *Measured GFR*

The mean (\pm standard deviation) GFR measured using blood spot iothexol clearance (BSIC-GFR) was 103.1 (\pm 28.3) ml/min/1.73m², significantly higher than that estimated by the MDRD formula (MDRD-GFR) and estimated from the blood concentration of cystatin c (cyc-GFR) (90.5 (\pm 18.1) and 92.0 (\pm 19.3) ml/min/1.73m² respectively) ($p < 0.001$ for each compared to BSIC-GFR) (Figure 3.7). In addition, the distribution of BSIC-GFR values was wider than that of the MDRD-GFR or the cyc-GFR with some individuals having very high BSIC-GFR values (Figure 3.7). The characteristics of participants with a “high” and “low” BSIC-GFR (defined as above the 95th centile or below the 5th centile of GFR measured by ¹²⁵iothalamate clearance among 365 healthy potential kidney donors (173)) are shown in table 3.2. As expected, participants with a “low” BSIC-GFR were older (mean \pm standard deviation age 67.7 \pm 9.8 compared to 52.4 \pm 13.5 among those with “normal” BSIC-GFR [$p < 0.001$]) and more likely to have cardiovascular disease (77% compared to 29% among those with “normal” BSIC-GFR [$p < 0.001$]) (Table 3.2). Surprisingly, the mean age among those with a “high” BSIC-GFR and those with a “normal” BSIC-GFR was similar (51.4 \pm 16.2 and 52.4 \pm 13.5 respectively [$0 = 0.794$]), as was the proportion of individuals with cardiovascular disease (38% and 29% respectively [$p = 0.479$]).

3.4 Discussion

3.4.1 Potential advantages of the BSIC method for use in epidemiological studies

In epidemiological studies, the use of dried capillary blood spots has important practical and economic advantages over venous blood samples; avoiding the need for trained phlebotomists, venepuncture equipment, refrigeration of samples and early laboratory processing (217). In the measurement of GFR, the use of these samples has the additional advantage that the collection of the timed blood samples could potentially be completed by the subjects themselves, substantially reducing the amount of time spent in the clinic.

In this study, a wide range of individuals were trained to take capillary blood samples during a single clinic visit, and all but 1 participant returned at least one dried capillary blood sample through the post. Both the injection of iohexol in the clinic and the finger-prick blood sample collection after the clinic were acceptable to > 90% of participants.

3.4.2 Methodological issues raised by this study

This study assesses two importance methodological issues in the development of the BSIC technique; the quality of samples collected by study participants at home and the accuracy of the recorded sample time.

The quality of the dried capillary blood spot samples may be important in determining the accuracy of the BSIC method. In order to estimate the concentration of any substance in a dried capillary blood spot, it is necessary to know the volume of blood

contained within the bloods spot sample. The method used in this study, as in other dried blood spot assays (221-224), makes use of the properties of the filter paper, which, provided the sample is collected using proper technique (a single drop of blood allowed to diffuse into the card by capillary action), can be assumed to contain an approximate volume of blood within a standard punch of the dried filter paper blood spot. The sample absorption properties of the filter papers are standardized under United States (US) Food and Drug Administration regulations and are subjected to a rigorous quality-control program by the US Newborn Screening Quality Assurance Program (218). However, the volume of blood contained in the standard punch is influenced by the blood haematocrit (225), the amount of blood dropped onto the card (218) and whether the punch is taken from the centre or the periphery of the blood spot (218). Layering of blood spots, when more than one spot applied to the same area of the card, and smudging of the blood onto the card with the finger could have substantial effects on the volume of blood contained in a standard punch. In this study, just less than one quarter of participants returned a blood spot at one or more time point which was judged to be 'poor quality'. This suggests that analysis of iohexol concentration in dried capillary blood spots collected by participants themselves using the current laboratory method may be not be accurate. Direct estimation of the volume of blood contained within a whole dried blood spot might enable more accurate measurement of iohexol concentration in poor quality blood samples for 2 reasons; First, it would not be necessary to rely on the assumed relationship between the area of the filter paper and the volume of blood contained within that area, which may not be valid in poor quality spots. Second, it would increase the total amount of sample which can be analysed, since it would not be necessary to

discard the 'un-punched' area. Such methods might include the simultaneous measurement of the quantity of a 'volume marker' (e.g. haemoglobin or sodium (225)) in the dried blood spot, measurement of the concentration of the same 'volume marker' in the baseline venous blood sample and, assuming that the concentration of the 'volume marker' in venous and capillary blood is the same, the volume of blood in the spot required could then be calculated. This study suggests that such advances in laboratory technique may be necessary to accurately measure the concentration of iohexol (and other parameters) in dried blood spots collected by study participants themselves.

The test accuracy could be affected by inaccuracies in the sampling time in 3 ways: The samples could be taken outside the scheduled time but the actual sampling time recorded accurately, the samples could be taken at the scheduled time but the sampling time recorded inaccurately, or the both errors could occur.

First, substantial deviation from the requested sampling time might reduce test accuracy even if the sampling time is recorded accurately. When blood spots samples are collected by laboratory staff, iohexol clearance measured using 2 dried capillary blood spots taken at 3 and 4 hours, or 2 and 3 hours after the iohexol injection is less accurate than that measured using samples taken at 2 and 4 hours (Section 2.3.7). In this study, the self recorded sampling time was within 15 minutes of the scheduled 2 and 4 hour sampling times in > 90% of the 108 participants who returned both samples.

Second, inaccurate recording of sampling time could reduce the accuracy of the blood spot iohexol clearance measurement. In this study, the true sample time was not known

and therefore the error in recording of the sample time cannot be assessed directly. However, this could be explored by examination of digit preference. Analysis of digit preference has suggested a tendency to 'round up' numeric data in a range of research settings, including the recording of blood pressure (226), self reported number of cigarette smoked per day (227) and in recorded attendance times in accident and emergency departments (228). In this study, analysis of the terminal digit of self reported sampling time demonstrated a preference for the digit "0" suggesting that the sample time was not accurately recorded in at least some participants. In chapter 2, modeling the impact of error in recorded sample time previously suggested that a scenario in which approximately a third of individuals recorded the sample time with an error of at least 15 minutes would have only a small effect on the accuracy of the BSIC method. However, there is some evidence that this apparent "rounding-up" of the sampling time may reflect more substantial inaccuracy in some participants. The finding that 15% of individuals in this study had a BSIC-GFR value above the 95th centile of GFR measured by ¹²⁵Iothalamate among 365 healthy potential kidney donors (mean age 41.1 years) is surprising since our study population is older (mean age 54.3 years) and 38% of participants had cardiovascular disease. In addition, those individuals with "high" BSIC-GFR values (those above the 95th centile of GFR among potential kidney donors) were not younger or less likely to have cardiovascular disease than those with a "normal" BSIC-GFR (those between the 5th and 95th centile of GFR among potential kidney donors). These findings suggest that some individuals might have forgotten to take one or more of the blood spot samples until after the scheduled time and then "made up" a sampling time near to the scheduled time resulting in artificially high

iohexol clearance values. The impact of this sort of error on test accuracy would be substantial. Providing participants with an automated time recording device must be considered for future studies.

3.4.3 Weaknesses of this study

Two weaknesses of this study should be considered. First, the study population was not a random sample of the local population. In addition, the reasons for not volunteering, which may include finding the idea of the BSIC procedure unacceptable, are not known. The recruitment methods were selected for pragmatic reasons and did ensure that the study population, the majority of which were not University Staff, included men and women across a wide range of age groups. Since the ages of the participants ranged from 24-77 years, these results may not be representative of the feasibility of undertaking this procedure in the elderly.

Second, since a reference GFR was not measured, the accuracy of the blood spot iohexol method in this setting cannot be directly assessed. Simultaneous measurement of a reference method using timed venous blood samples would invalidate the attempt to assess the feasibility of conducting the procedure outside the clinic environment.

Subsequent measurement of a reference method GFR would have substantially increased the resources required for the study, both in cost and staff time and would have required an additional visit to the clinic for the participants. The aim of this study was to assess the feasibility of measuring blood spot iohexol clearance from samples collected by

participants themselves after the clinic to inform further method development. Further validation of the method would be required prior to use in an epidemiological study.

3.5 Summary of key findings

- Measurement of Blood Spot Iohexol Clearance (BSIC) using timed finger-prick blood samples collected after the clinic is acceptable to a wide range of individuals.
- The BSIC method is potentially feasible for use in large scale epidemiological studies, but laboratory techniques must be improved to make use of small and ‘poor quality’ blood spots.
- When the timed capillary blood sampling is completed by participants at home, the self reporting sampling time may be inaccurately reported. This study also suggests that in some participants there may be large discrepancies between the recorded and actual sampling times resulting in reduced test accuracy. Future studies should provide participants with an alarm and timing device.
- In view of the methodological issues raised by this study, further development and validation of the blood spot iohexol clearance technique is required.

Chapter 3

Tables and figures

Table 3.1: Characteristics of eligible participants

	University staff	SEARCH clinic	Oxford Kidney Unit	Total
n	43	53	15	111
Age	41.4±8.8 (25-58)	67.4±8.6 (40-77)	58.1±14.0 (40-76)	54.8±14.4 (24-77)
Female	56%	34%	60%	46%
Prior CVD	0%	75%	13%	38%
Diabetes	0%	8%	0%	4%
Hypertension	5%	28%	20%	18%
Current smokers	2%	8%	13%	6%
Ex-smokers	24%	62%	20%	42%

SEARCH, Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine; CVD, cardiovascular disease (defined as a self reported history of angina, myocardial infarction, coronary or non-coronary revascularisation, stroke or peripheral vascular disease).

Data are given as mean ± standard deviation (range) or percentage

Table 3.2: Characteristics of participants with a “high”, “low” and “normal” GFR measured by blood spot iothexol clearance

BSIC GFR (ml/min/1.73m ²)	low < 70	<i>p</i> *	normal 70-133	high >133	<i>p</i> **
n	13		77	16	
BSIC GFR (ml/min/1.73m ²)	58.3±12.1	<i>p</i> <0.001	100.9±15.0	151.1±13.8	<i>p</i> <0.001
MDRD GFR (ml/min/1.73m ²)	67.0±13.5	<i>p</i> <0.001	92.2±16.3	101.7±13.0	<i>p</i> =0.030
Cystatin c GFR (ml/min/1.73m ²)	65.0±12.8	<i>p</i> <0.001	93.4±16.1	107.8±15.8	<i>p</i> =0.002
Age (years)	67.7±9.8	<i>p</i> <0.001	52.4±13.5	51.4±16.2	<i>p</i> =0.794
Female	4 (31%)	<i>p</i> =0.247	37 (48%)	9 (56%)	<i>p</i> =0.551
Prior CVD	10 (77%)	<i>p</i> =0.001	22 (29%)	6 (38%)	<i>p</i> =0.479
Hypertension	5 (38%)	<i>p</i> =0.035	11 (14%)	3 (18%)	<i>p</i> =0.650

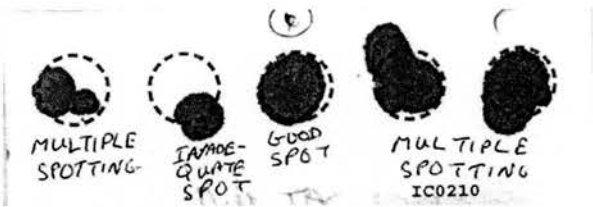
Data are shown as mean ± standard deviation or number (percentage). The cut points used to define a “low” and “high” GFR represent the 5th and 95th centile of GFR measured by ¹²⁵iothalamate clearance among 365 healthy potential kidney donors (173). Analysis is restricted to individuals who returned 2 analysable blood spot iothexol samples and had measurements of blood creatinine and cystatin c concentrations (n=106).

* Proportion or mean among those with “low” GFR compared to “normal” GFR compared using a chi squared test or 2 sample t-test respectively

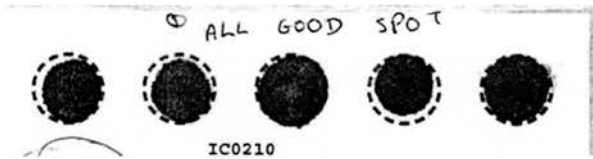
**Proportion or mean among those with “high” GFR compared to “normal” GFR compared using a chi squared test or 2 sample t-test respectively

BSIC, blood spot iothexol clearance; MDRD, modification of diet in renal disease study; CVD, cardiovascular disease

Figure 3.1: Example blood spots



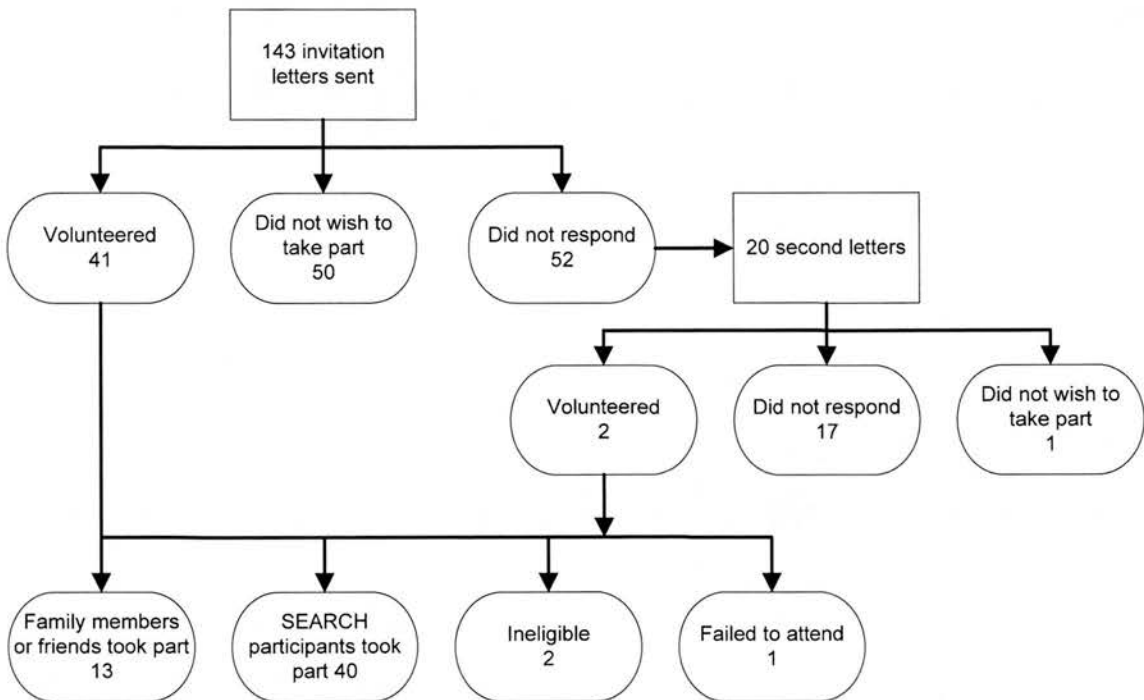
a. Examples of poor quality blood spots



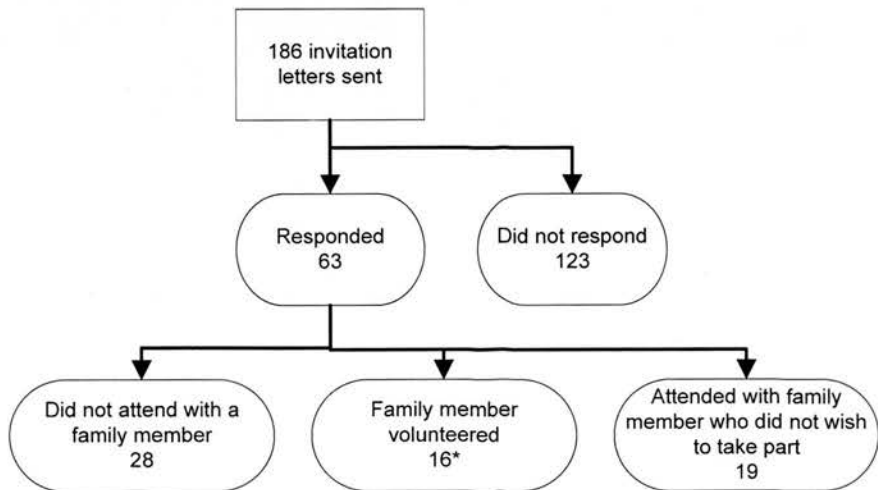
b. Examples of good quality blood spots

Figure 3.2: Recruitment of participants

a. Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH)

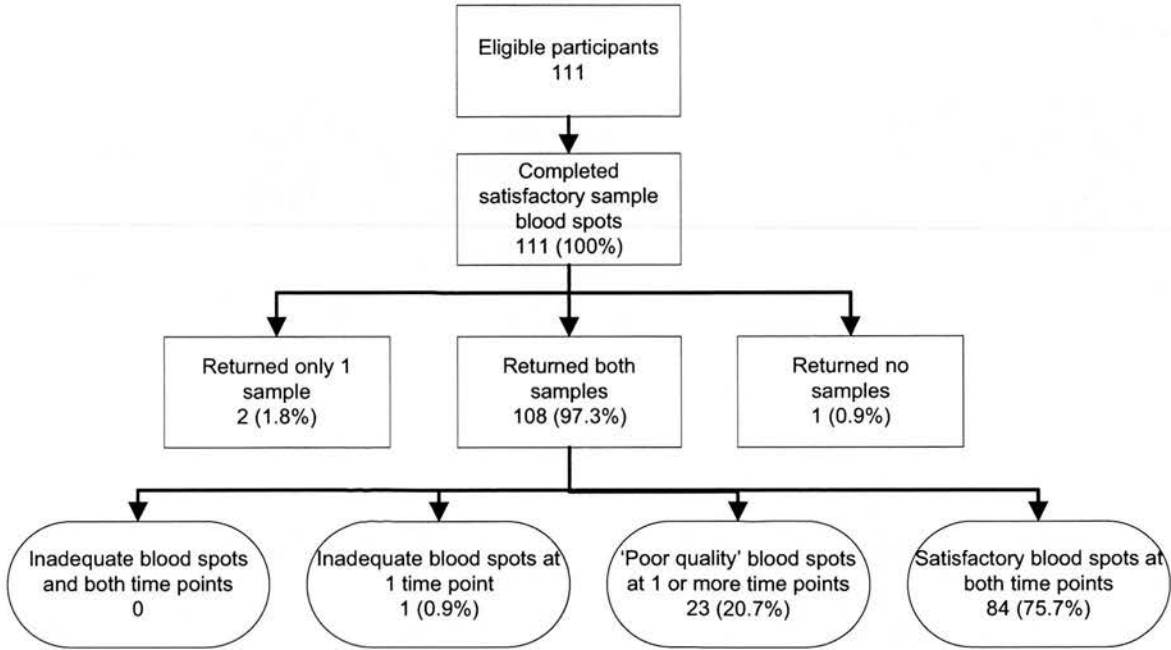


b. Oxford Kidney Unit (OKU)



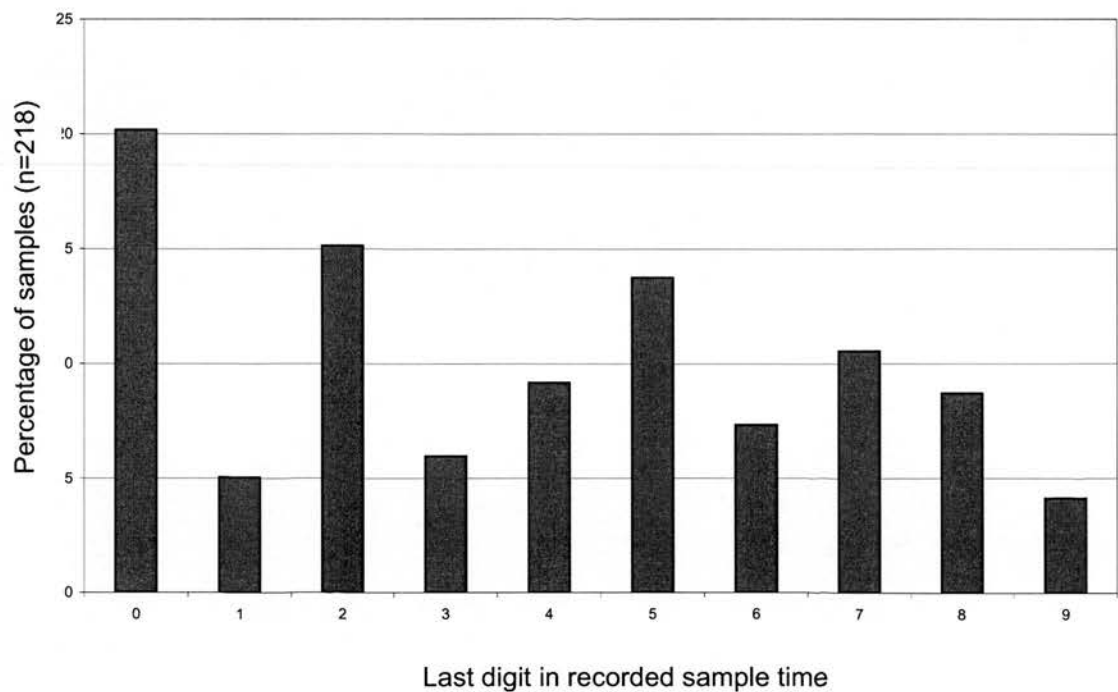
* 1 did not take part for administrative reasons

Figure 3.3: Number and quality of returned blood spot samples



A blood spot sample was considered ‘inadequate’ if no quantification of the concentration of iohexol was possible. A blood spot sample was considered ‘poor quality’ if there was evidence of poor collection technique (section 3.3.2).

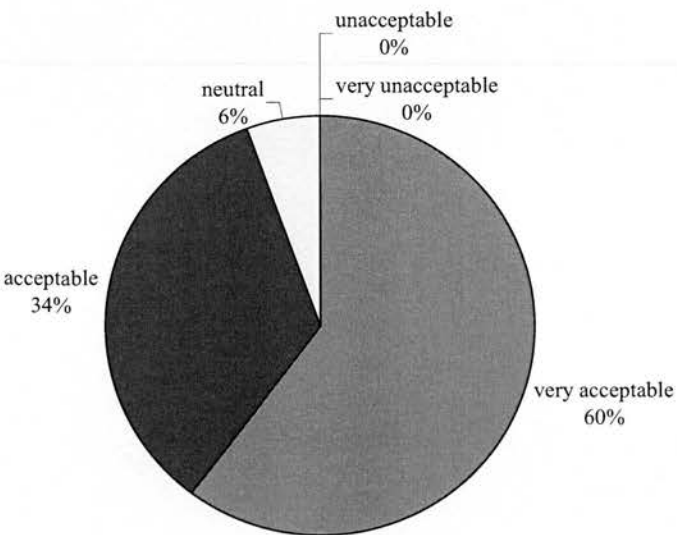
Figure 3.4: Frequency of the last digit used in the recorded blood spot sample time



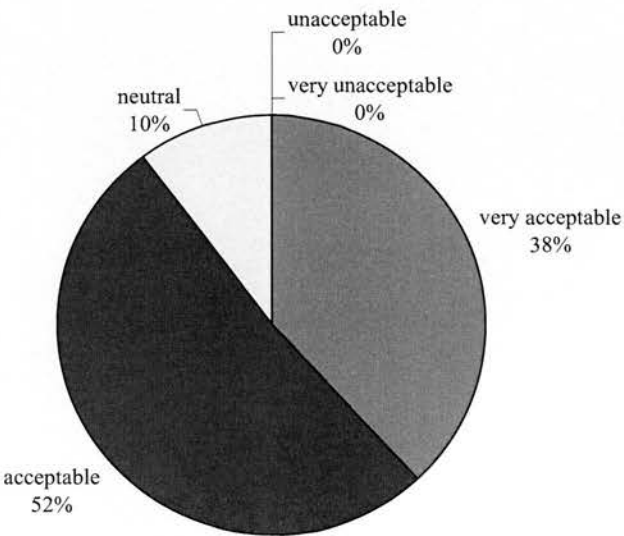
($p < 0.0001$, χ^2 comparison with an expected 10% frequency of each digit)

Figure 3.5: Acceptability of the finger-prick blood sample collection procedure to participants

a. “I found the iohexol injection at the clinic to be:”



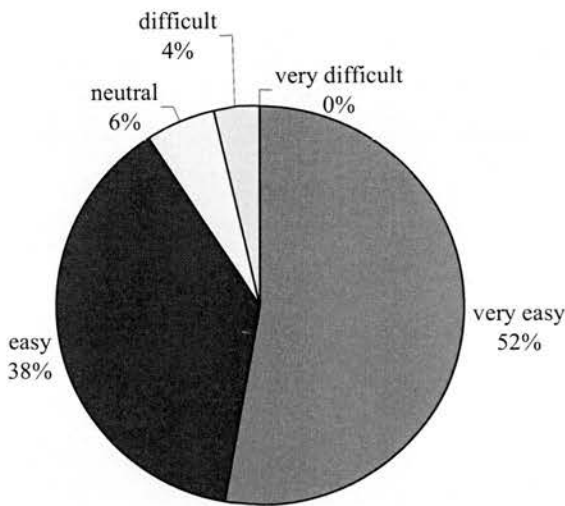
b. “Overall, I found taking the finger prick blood spots to be:”



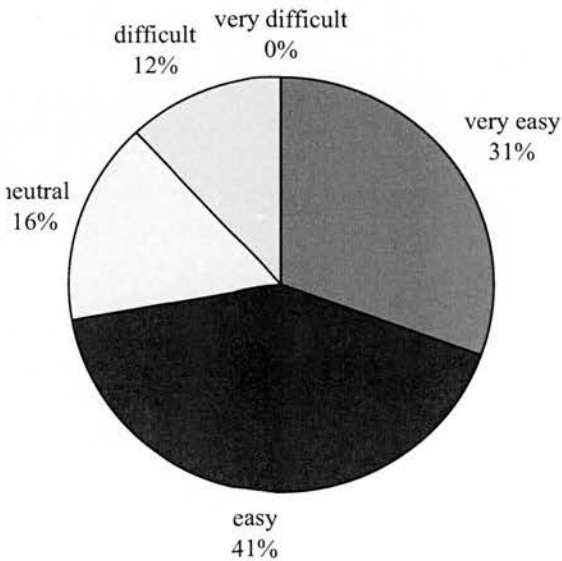
Data displayed as the proportion of respondents (n=108) who selected each answer on the questionnaire

Figure 3.6: Participants assessment of the finger-prick blood collection procedure

a. “In my experience, actually pricking the finger using the needle was:”

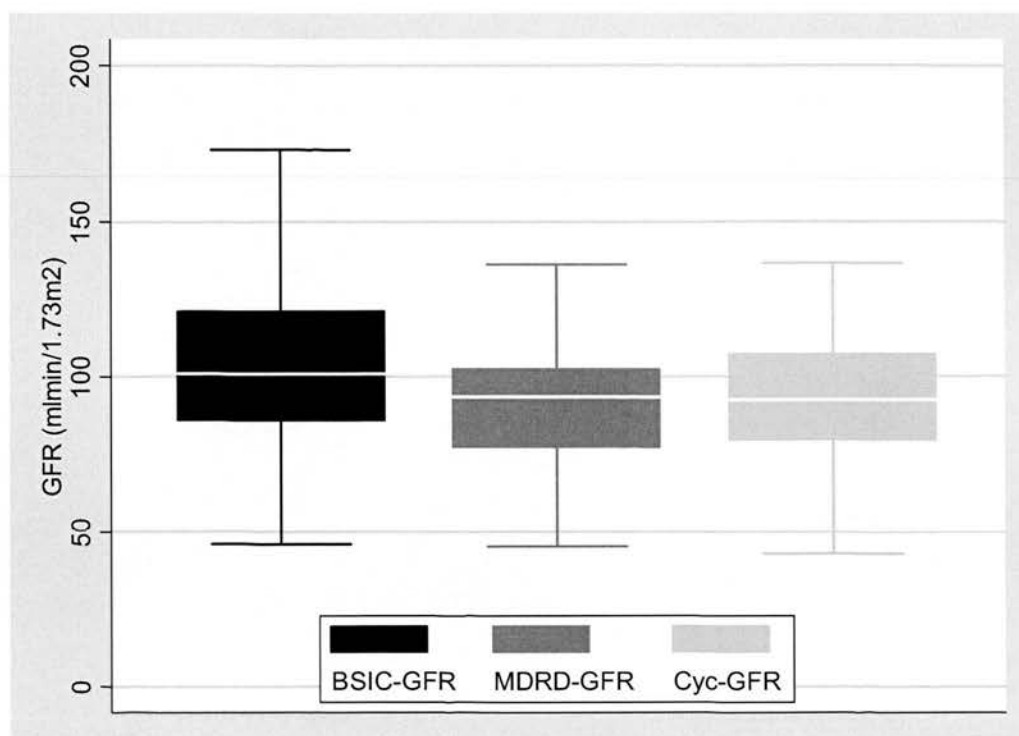


b. “I found dropping the blood spots onto the filter paper to be:”



Data displayed as the proportion of respondents (n=108) who selected each answer on the questionnaire

Figure 3.7: Box and whisker plots showing the distributions of glomerular filtration rate measured by blood spot iohexol clearance and estimated from blood creatinine and cystatin c concentrations



The central white lines indicate the median value, the grey boxes indicate the inter-quartile range and the bars indicate the 95% range.

BSIC, blood spot iohexol clearance; GFR, glomerular filtration rate; MDRD, modification of diet and renal disease formula; Cyc-GFR, GFR estimated from blood cystatin c concentration

Analysis is restricted to individuals who returned 2 analysable blood spot iohexol samples and had measurements of blood creatinine and cystatin c concentrations (n=106).

Chapter 4: Glomerular filtration rate and risk factors for cardiovascular disease

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4.1 Study aims

In the Chronic Renal Failure in Birmingham study, nephrology outpatients with an elevated serum creatinine ($>130 \mu\text{mol/l}$) had lower blood HDL-cholesterol concentrations and higher blood concentrations of triglyceride, homocysteine, fibrinogen and C reactive protein, compared to healthy controls (5;54). Furthermore, among those with chronic kidney disease, a graded relationship was observed between lower GFR (assessed by higher blood concentration of creatinine or cystatin c) and lower blood concentration of HDL-cholesterol and higher blood concentration of homocysteine and fibrinogen (5;54). We aimed to extend these findings by examining the cross sectional relationships between measures of GFR (including an exogenous tracer based method) and cardiovascular risk factors in a sample of individuals without chronic kidney disease.

This study aims to;

1. examine the cross-sectional relationships between measures of GFR and the following risk factors for cardiovascular disease

- (i) blood pressure
- (ii) blood concentration of lipids and apolipoproteins,
- (iii) blood concentration of fibrinogen, C reactive protein and homocysteine
- (iv) markers of body composition

2. assess how these relationships are affected by the method used to measure GFR (blood spot iohexol clearance, creatinine-based estimates of GFR, or GFR calculated from serum cystatin C concentration).

Methods

This study was conducted among volunteers taking part in the pilot study described in chapter 3. The recruitment methods, participant characteristics and data collection techniques are described in section 3.2. Participants were included in this study if they had no missing values for GFR measured by blood spot iohexol clearance and estimated from blood concentrations of cystatin c and creatinine (n=106). Thirty six percent of the participants in this study were receiving either homocysteine lowering treatment (folic acid and vitamins B6 and B12) or placebo as part of the double blinded Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH). Such treatment was recorded as 'possible folic acid'.

4.1.1 Laboratory methods

Details of the blood spot collection and storage methods are given in sections 3.2.3.7 and 3.2.3.9. The methods used to analyse blood iohexol concentration in dried capillary blood spots, and the calculations used to estimate GFR from 2 spots are outlined in section 2.2.3.1. Blood samples were collected as outlined in section 3.2.3. Whole blood was removed from the EDTA sample for analysis of haematocrit and haemoglobin. After centrifuging, aliquots of plasma were removed from the EDTA sample for analysis of concentration of creatinine, cystatin c, total cholesterol, High Density Lipoprotein

(HDL)-cholesterol, Low Density Lipoprotein (LDL)-cholesterol, triglycerides, apolipoproteins A₁ and b, C-reactive protein and fibrinogen, and an aliquot of plasma removed from the sodium fluoride EDTA blood sample for analysis of homocysteine. The remaining EDTA and sodium fluoride EDTA plasma samples were transferred into cryovials and stored at -80°C for future use. GFR was calculated from blood creatinine concentration by the abbreviated MDRD formula (Table 1.2) and from blood cystatin c concentration by the formula by Hoek et al (Table 1.5). Eighty eight percent of samples were processed on the same day as collection with the remaining samples processed the following day. Table 4.1 provides details of the laboratory methods used in this study.

4.1.2 Statistical methods

Data was analysed using Stata v8.2 (StataCorp, Texas, USA). Continuous variables were assessed for normality by visual inspection of a normal plot and by a Shapiro-Wilk test. Variables which were not normally distributed (blood HDL-cholesterol, LDL-cholesterol, total-cholesterol, apolipoprotein A₁, apolipoprotein b and triglyceride concentrations, weight, and body mass index) were transformed by taking a logarithm to the base 10 and the log[variable] then tested for normality. The normal plot was also be used to visually assess for outliers. The value of any outlier more than 3 standard deviations away from the mean was double checked against the source data and, if there was reason to believe that the value was spurious, it was excluded. For the analysis involving C-reactive protein (CRP), one participant with an outlying value of 47 mg/L was excluded since this value was likely to reflect an acute rise in CRP. Normally distributed variables were expressed as mean \pm standard deviation. Variables which

were not normally distributed were expressed as median (interquartile range).

Differences in proportions across tertiles of GFR were compared by a chi squared test for trend while differences in mean values across tertiles of GFR were compared using ANOVA test for trend (using the geometric mean if the variable was not normally distributed). Pearson correlations and least squares linear regression was used to examine relationships between continuous variables. No quantitative adjustments for multiple testing were performed but due allowance for this was made in the interpretation of the results.

4.2 Results

4.2.1 *Participant characteristics*

The mean±standard deviation GFR measured using blood spot iothexol clearance (BSIC-GFR), estimated by the MDRD formula (MDRD-GFR) and estimated from the blood concentration of cystatin c (cyc-GFR) was 103.1±28.3, 90.5±18.1 and 92.0±19.3 ml/min/1.73m² respectively and the distributions of these GFR measures are shown in chapter 3, figure 3.7.

The participant characteristics and cardiovascular risk factors by tertiles of BSIC-GFR are shown in table 4.2. Those with lower BSIC-GFR were older, more likely to have a prior history of cardiovascular disease and were more likely to be receiving lipid, cholesterol or homocysteine lowering medication. All 3 GFR measures correlated strongly with age ($r=-0.31$ [$p=0.002$] for BSIC-GFR, $r=-0.40$ [$p<0.001$] for MDRD-GFR and $r=-0.49$ [$p<0.001$] for Cyc-GFR).

4.2.2 Measures of GFR and 'traditional' cardiovascular risk factors

No correlations could be detected between any of the GFR measures and diastolic blood pressure, systolic blood pressure, or blood concentration of total-cholesterol, LDL-cholesterol or apo-lipoprotein b. Summary statistics describing the relationships between blood concentrations of HDL-Cholesterol, apolipoprotein A₁ and triglyceride, and each of the GFR measures are shown in table 4.3. Scatter plots showing the relationship between the GFR measures and blood triglyceride concentration are shown in figure 4.1.

4.2.2.1 Blood Spot Iohexol Clearance GFR (BSIC-GFR) and blood lipid concentrations

The correlations between BSIC-GFR and blood concentration of HDL-cholesterol and apolipoprotein A₁ were not statistically significant ($r=0.167$ [$p=0.087$] and $r=0.083$ [$p=0.397$] respectively). However, a correlation was observed between blood triglyceride concentration and BSIC-GFR ($r=-0.247$ [$p=0.011$]. After adjustment for age and sex, a 30 ml/min/1.73m² lower BSIC-GFR was associated with 4.9% higher blood triglyceride concentration, although the 95% confidence interval (CI) for this estimate (1.1% lower to 11.0% higher) crossed zero (Table 4.3).

4.2.2.2 MDRD-eGFR and blood lipid concentrations

Blood concentration of HDL-cholesterol and apolipoprotein A₁ were not significantly correlated with MDRD eGFR ($r=0.136$ [$p=0.166$] and $r=0.075$ [$p=0.113$] respectively). A relationship was observed between MDRD eGFR and blood triglyceride concentration ($r=-0.278$ [$p=0.004$]). After adjustment for age and sex, a 30 ml/min/1.73m² lower

MDRD-eGFR was associated with an 11.5% (95% CI 1.6 to 21.3%) higher blood triglyceride concentration (Table 4.3).

4.2.2.3 Cystatin C eGFR (cyc-eGFR) and blood lipid concentrations

Cyc-eGFR was correlated with blood concentration of HDL-cholesterol and triglycerides ($r=0.390$ [$p<0.001$] and $r=0.392$ [$p<0.001$] respectively). A similar correlation was seen between cyc-eGFR and apolipoprotein A₁ $r=0.283$ [$p=0.003$]. After adjustment for age and sex, a 30 ml/min/1.73m² lower cyc-eGFR was associated with a 6.5% (95% CI 2.7-10.3%) lower blood concentration of HDL cholesterol, a 3.8% (95% CI 1.5-6.2%) lower blood apolipoprotein A₁ concentration and a 14% (95% CI 4.2-24%) increase in blood triglyceride concentration (Table 4.3).

4.2.3 Measures of GFR and novel cardiovascular ‘biomarkers’

Summary statistics describing the relationships between blood concentrations of homocysteine, CRP and fibrinogen and the 3 measures GFR are shown in table 4.4. Scatter plots showing the relationship between the GFR measures and blood homocysteine concentration are shown in figure 4.2.

4.2.3.1 BSIC-GFR and novel cardiovascular ‘biomarkers’

No correlation between BSIC-GFR and the blood concentrations of CRP or fibrinogen could be detected ($r = -0.149$ [$p=0.128$], $r=-0.141$ [$p=0.150$] respectively, table 4.4). However, an inverse correlation between BSIC-GFR and blood homocysteine concentration was observed ($r=-0.415$ [$p<0.001$], table 4.4). After adjustment for age and sex, a 30 mlmin/1.73m² lower BSIC-GFR was associated with a 5.3% (95% CI 3-7.4%) higher blood homocysteine concentration (Table 4.4).

4.2.3.2 MDRD-GFR and novel cardiovascular ‘biomarkers’

No correlation between MDRD GFR and blood concentration of CRP could be detected ($r=0.140$ [$p=0.151$], table 4.4). However, MDRD-eGFR correlated with blood concentration of both fibrinogen ($r=-0.378$ [$p<0.001$]) and homocysteine ($r=-0.414$ [$p<0.0001$]) (Table 4.4). After adjustment for age and sex, a 30 mlmin/1.73m² lower MDRD-GFR was associated with a 0.21 g/l (95% CI 0.02-0.40 g/l) higher blood fibrinogen concentration and a 9.8% (95% CI 6.4-13.4%) higher blood concentration of homocysteine (Table 4.4).

4.2.3.3 Cyc-GFR and novel cardiovascular 'biomarkers'

A correlation was observed between cystatin C eGFR and blood concentrations of CRP ($r=-0.304$ [$p=0.002$]), fibrinogen ($r=-0.404$ [$p<0.001$]) and homocysteine ($r=-0.422$ [$p<0.0001$]) (Table 4.4). After adjustment for age and sex, a 30 ml/min/1.73m² higher cyc-eGFR was associated with a 19.6% (95% CI 5-34.3%) increase in blood concentration of CRP, a 10.0% (95% CI 6.3-13.6%) increase in blood homocysteine concentration and a 0.28 g/l (95% CI 0.09-0.47 g/l) higher blood fibrinogen concentration.

4.2.4 Measures of GFR and markers of body composition

Summary statistics describing the relationship between the 3 GFR methods and body mass index (BMI), waist circumference and percentage body fat measured by bioimpedence are shown in table 4.5. Scatter plots showing the relationship between measures of GFR and waist circumference are shown in figure 4.3.

No correlation could be detected between BSIC-GFR and BMI, waist circumference or percentage body fat (Table 4.5). Similarly, no correlation could be detected between MDRD-GFR and either BMI or waist circumference. MDRD-GFR was negatively correlated with percentage body fat ($r=-0.307$ [$p=0.002$]) but after adjustment for age and sex, lower MDRD-GFR was not associated with greater body fat (β coefficient 0.043 per ml/min/1.73m² lower MDRD-GFR [$p=0.284$], table 4.5).

Cyc-GFR, however, was negatively correlated with BMI ($r=0.330$ [$p=0.001$]), waist circumference($r= -0.507$ [$p<0.001$]) and percentage body fat ($r=-0.242$ [$p=0.014$]). After adjustment for age and sex, a 30 ml/min/1.73m² lower cyc-eGFR was associated with a 2.7% (95% CI 0.1-5.3%) higher BMI, a 6.1cm (95% CI 2.4-9.9 cm) larger waist and 3.5% (95% CI 1.2-5.8%) greater body fat (Table 4.5).

4.3 Discussion

This study demonstrates that, in a small group of 24 to 77 year olds, measures of GFR were positively correlated with blood HDL-cholesterol and apolipoprotein A₁ concentrations, and were inversely correlated with blood concentrations of triglyceride, CRP, fibrinogen, and homocysteine.

4.3.1 *Potential clinical relevance of these associations*

After adjustment for age and sex, a 30 ml/min/1.73m² lower GFR was associated with a 0-7% lower blood HDL-cholesterol concentration, a 5-14% higher blood triglyceride concentration, a 0-20% higher blood concentration of CRP, a 5-10% higher blood homocysteine concentration and a 0-0.28 g/l higher blood fibrinogen concentration, depending on the GFR measure examined (Tables 4.3 and 4.4). While modest, some of these changes in cardiovascular risk factors could be associated with clinically relevant increases in cardiovascular risk.

In observational studies a 0.1 mmol/l decrease in HDL cholesterol (approximately equal to a 7% decrease in this study) was associated with a 20% increased risk of coronary heart disease (48). For triglycerides, a 1 mmol higher blood concentration is associated with a 14% and 37% increased risk of cardiovascular disease in men and women respectively in observational studies (50). Therefore, a 14% increase in blood triglyceride concentration in this study (approximately equal to 0.15 mmol/l higher blood concentration) might be associated with a 1.02-1.06 fold increase risk of cardiovascular disease. In observational studies an increase in CRP of around 1.4 mmol/l

is associated with a 1.9 fold increase in risk of coronary artery disease (51). Therefore a 20% higher CRP concentration in this study (approximately equal to only a 0.16 mg/l) might be associated with roughly a 1.1 fold increase in risk of coronary artery disease. In observational studies, a 25% higher blood homocysteine concentration is associated with a 1.11 fold higher risk of ischaemic heart disease (69), while a 1g/l higher blood fibrinogen concentration is associated with a 1.8 fold increase risk of coronary disease (55). Therefore, a 10% higher blood homocysteine concentration and 0.28 g/l higher blood fibrinogen concentration might be associated with a 1.04 fold and 1.22 fold increase in ischaemic heart disease risk respectively. If these risks were additive one might expect the change in risk factors accompanying a 30 ml/min/1.73m² lower GFR to be associated with a small, but potentially clinically relevant, increase in the risk of cardiovascular disease.

4.3.2 Possible pathophysiological basis for these associations

This study demonstrates a relationship between measures of lower GFR and higher blood concentrations of triglycerides, CRP, fibrinogen, and homocysteine, and lower blood concentrations of HDL-cholesterol and apolipoprotein A₁. In this cross-sectional study, it is not possible to explore whether these adverse cardiovascular risk factors might be a cause or a consequence of lower GFR. However, a number of lines of evidence suggest that substantial loss of GFR in chronic kidney disease (CKD) may result in dyslipidaemia, a pro-inflammatory state and increased blood homocysteine concentrations.

ESKD is associated with impaired clearance of triglyceride rich apolipoprotein B containing very low density lipoprotein (VLDL) and intermediate density lipoprotein (IDL) particles (32;43) (Figure 4.4). These abnormalities are probably the result of decreased activity of the key enzymes which regulate the metabolism of blood triglycerides, lipoprotein lipase (LP) and hepatic lipase (HL) (Figure 4.4) (32;46;49). In addition, changes in the composition of VLDL particles, particularly increased surface apolipoprotein C_{III}, in ESKD (47) may further impair their clearance (46). The dyslipidaemia of ESKD is also characterised by impaired maturation of HDL particles (32;46) resulting in low blood concentration of the mature HDL₂ particles (49) (Figure 4.4). Reduced activity of the enzyme lecithin cholesterol acetyltransferase (LCAT) in ESKD is likely to be at least partly responsible for this abnormal reverse cholesterol transport (25;32). Evidence from experimental models of CKD in animals suggests a causal relationship between loss of GFR and these lipid abnormalities (32).

Experimental CKD is associated with reduced activity and tissue gene expression of LP (229;230), HP (231) and LCAT (232;233) and decreased hepatic production of apolipoprotein A₁ (32) (Figure 4.5). Evidence suggests that these abnormalities in lipid metabolism occur early in the natural history of CKD. In the Chronic Renal Failure in Birmingham (CRIB) study, blood HDL cholesterol was 13% lower and blood triglyceride concentration 58% higher among those with CKD (defined as nephrology clinic outpatients with a creatinine >130 µmol/l) compared to age and sex matched healthy controls (5). In a cross sectional analysis from the Cardiovascular Health Study, a population based cohort of individuals aged ≥65 years in the United States, individuals with CKD (defined as a serum creatinine concentration >133 µmol/l in men or >115 µmol/l in women) had higher blood HDL-cholesterol and lower blood triglyceride concentrations than those without CKD (59).

Compared to healthy controls, individuals with ESKD have substantially higher blood concentrations of CRP (53) and fibrinogen (56). In the CRIB study, blood concentrations of fibrinogen and CRP were 0.8g/L and 1.5mg/L higher respectively among those with CKD than age and sex matched controls (54), and, in the Cardiovascular Health Study, CKD was associated with higher blood concentrations of inflammatory markers (CRP, fibrinogen and interleukin-6) after adjustment for age, sex and other cardiovascular risk factors (59). Potential reasons for these findings include reduced clearance (234) or increased production as a result of systemic inflammation (235;236). It is increasingly recognised that CKD is associated with a state of generalised systemic inflammation (235;236). Postulated reasons for this include an

effect of renal disease itself, the result of other associated conditions (such as diabetes or cardiovascular disease), or, among those with ESKD, pro-inflammatory effects of the dialysis treatments per se (52;235). A number of mechanisms by which renal disease might cause increased inflammation have been postulated, including the reduced clearance of pro-inflammatory cytokines (235) and the accumulation of advanced glycosylation end-products (AGE) (237-239).

Blood homocysteine concentration is markedly elevated among individuals with CKD (27). In one study, blood homocysteine concentration was 22.7 $\mu\text{mol/l}$ among 25 individuals receiving haemodialysis therapy, compared to 9.5 $\mu\text{mol/l}$ among 25 age and sex matched controls drawn from the Framingham study (240). In the CRIB study plasma homocysteine concentration was 20.6 $\mu\text{mol/l}$ among 369 individuals with CKD, compared to 10.0 $\mu\text{mol/l}$ among 103 age and sex matched controls (5). Among those with CKD, plasma homocysteine concentration was strongly correlated with blood creatinine concentration (5). Among 109 individuals attending a nephrology clinic with a serum creatinine concentration $<130 \mu\text{mol/l}$ and GFR 18-205 ml/min/1.73m^2 measured by $^{51}\text{Cr-EDTA}$ clearance, blood homocysteine concentration showed a strong inverse correlation with GFR independent of age, blood concentration of folate, vitamin B12 and vitamin B6, and proteinuria (partial $r = -0.408$) (70). A similar strong and graded relationship between blood homocysteine concentration and GFR has been demonstrated among the elderly (27), patients with coronary artery disease (241) and individuals with diabetes mellitus (242). The mechanism by which homocysteine accumulates in CKD remains unclear (243). Clearance of homocysteine from the plasma is markedly reduced

in CKD (244) and, in dialysis patients, plasma homocysteine concentration fall rapidly following renal transplantation (245). However, reduced excretion of homocysteine in the urine is unlikely to be responsible. Excretion of homocysteine in the urine in health is minimal (246;247) and, although studies in rats have suggested that homocysteine may be metabolised by renal tubular cells (248), human studies have failed to support this hypothesis (249). Extra-renal effects of uraemic toxins on homocysteine metabolism have been postulated, this may not explain the strong relationship seen between plasma homocysteine concentration and GFR among individuals with a relatively preserved renal function (243).

4.3.3 Impact of choice of GFR measure

Since a large number of statistical comparisons were made in this exploratory study, small differences in the β regression coefficient or the correlation coefficient should not be over interpreted. However, two key points should be noted.

First, the relationships between BSIC-GFR and blood HDL-cholesterol apolipoprotein A₁, triglyceride, CRP, fibrinogen and homocysteine concentrations were not materially stronger than the relationships between the MDRD-GFR and these risk factors. Since GFR estimated from the MDRD formula is only weakly related to true GFR among individuals without CKD (20;174), one would expect GFR measured using an exogenous tracer based method to be more closely related to cardiovascular risk factors. One could speculate that, since the distribution of BSIC-GFR was wider than that of MDRD-GFR (Figure 3.7), the difference in cardiovascular risk factors associated per 1

ml/min/1.73m² might not be comparable. However, expressing the β coefficients of regression analysis per standard deviation increase in GFR measure did not materially alter the results. Blood spot iohexol clearance accurately reflects traditional iohexol clearance measured using timed venous blood samples when samples are collected by trained personnel (Section 2.3.2). However, in this study BSIC-GFR was measured using dried capillary blood spots collected by participants themselves at home as part of a pilot study assessing the feasibility of the technique. Twenty two percent of participants returned 'poor quality' spots and there was evidence that, not only was the sampling time recorded inaccurately in a proportion of individuals, but that large discrepancies between the actual and recorded sampling time may have occurred (Section 3.4.2). Therefore, it is likely that error in the BSIC-GFR method resulted in a weaker relationship between BSIC-GFR and cardiovascular risk factors than would be observed if true GFR was measured.

Second, the relationships between Cyc-GFR and BMI, waist circumference and percentage body fat measured by bioimpedance, were substantially stronger than that seen with either BSIC-GFR or MDRD-GFR after adjustment for age and sex. The relationship between increased body fat and GFR is complex. In cross-sectional studies, higher BMI is associated with a normal or increased GFR but increased renal plasma flow to GFR ratio indicating hyper-filtration (250;251). Individuals with higher BMI, particularly those with central obesity, are also more like to have microalbuminuria (252). Histological examination of renal tissue from obese kidney donors and animals fed a high fat diet show structural glomerular changes, particularly glomerular expansion,

compared to non-obese controls (253;254). These early changes associated with obesity may lead on to glomerulosclerosis and progressive renal damage and in prospective studies, higher BMI is associated with an increased risk of developing chronic kidney disease (255;256) and end stage renal failure (257) decades later. However, some studies have suggested that blood cystatin c concentration might be related to body composition independent of GFR. In a large community sample, weight was related to blood cystatin c concentration after adjustment for measured creatinine clearance (141). In addition, among individuals with CKD, lean body mass was related to blood cystatin c concentration independent of inulin clearance (258). One explanation of these, and our findings, could be increased cystatin c production among those with greater body fat. In addition, in this study, cyc-GFR tended to be more strongly associated with blood lipids and inflammatory markers, particularly blood HDL-cholesterol and CRP concentrations, than BSIC-GFR or MDRD-GFR (Tables 4.3 and 4.4). Cystatin c is increasingly employed to assess GFR in large scale epidemiological studies. Larger samples which assess a reference GFR, blood cystatin c concentration and cardiovascular risk factors, including assessment of body composition, are needed to properly determine the non-GFR determinants of blood cystatin c concentration.

4.3.4 Weaknesses of the study

Three main weaknesses of this study should be considered. First, the study included only 106 individuals. The failure to detect relationships, for example, between markers of GFR and blood pressure, could result from a lack of statistical power.

Second, this study may underestimate the strength of the relationships between measures of GFR and blood concentrations of lipids and homocysteine since those with lower GFR were more likely to be taking lipid lowering medication or folic acid (Table 4.2). Similarly, the failure to detect a relationship between measures of GFR and blood pressure probably results from the greater antihypertensive use among those with lower GFR (Table 4.2).

Third, since the data was collected as part of a pilot study to assess the feasibility of using the blood spot iothexol clearance technique in which participants collect the finger-prick capillary blood samples themselves at home, no ‘gold-standard’ GFR was measured. The aim of this pilot study was to explore the relationships between the difference measures of GFR and cardiovascular risk factors while assessing the feasibility of the BSIC method. Larger scale studies in which a fully validated GFR method is measured would enable further assessment of the relationship between GFR and cardiovascular risk factors and also, with long term follow-up, determine the true impact of GFR on the cardiovascular outcomes and death.

4.4 Summary of key findings

- In this sample, measures of GFR were positively correlated with blood HDL-cholesterol and apolipoprotein A₁ concentrations, and were inversely correlated with blood concentrations of triglyceride, CRP, fibrinogen, and homocysteine.
- The magnitude of the change in these cardiovascular risk factors accompanying a 30ml/min1.73m² lower GFR could be associated with a small, but potentially clinically relevant, increase in risk of cardiovascular disease.
- GFR measured by blood spot iohexol clearance was not more strongly related to cardiovascular risk factors than that estimated from the MDRD formula, probably as a result of error in the BSIC-GFR method used in this study.
- GFR estimated from blood cystatin c concentration tended to be more strongly correlated with blood lipids and inflammatory markers than GFR measured by the other two methods.
- Measures of body fat are strongly correlated with GFR estimated from cystatin c concentration, but not with either GFR estimated from the MDRD equation or measured using blood spot iohexol clearance.

Chapter 4

Tables and figures

Table 4.1: Details of laboratory methods

Parameter	Sample	Additive	Analyser (make, company county, country)	Reagents (company, county, country)	Between run CV
Haematocrit	Whole blood	EDTA	GEN-S, Beckman Coulter, Bucks, UK.	Beckman Coulter	1%
Haemoglobin	Whole blood	EDTA	As above	As above	1%
Total-Cholesterol	Plasma	EDTA	Synchron LX20, Beckman Coulter, Bucks, UK.	Beckman Coulter	2%
HDL-Cholesterol	Plasma	EDTA	As above	Ultra N-geneous HDL, Bio-Stat Limited, Cheshire, UK.	3%
LDL-Cholesterol	Plasma	EDTA	As above	N-geneous LDL, Bio-Stat Limited, Cheshire, UK.	3%
Triglyceride	Plasma	EDTA	As above	Beckman Coulter	4%
Apolipoprotein A ₁	Plasma	EDTA	As above	As above	3%
Apolipoprotein B	Plasma	EDTA	As above	As above	5%
Creatinine	Plasma	EDTA	As above	As above	7% at 40 $\mu\text{mol/l}$ 1% at 358 $\mu\text{mol/l}$
Cystatin C	Plasma	EDTA	BNII automated nephelometer, Dade-Behring, Bucks, UK.	Dade-Behring	5%
C-Reactive protein	Plasma	EDTA	As above	Cardio phase high sensitivity CRP, Dade Behring	4%
Fibrinogen	Plasma	EDTA	As above	Dade Behring	4%
Homocysteine	Plasma	Na-F EDTA	HPLC, Waters, Herts, UK.	In house (259)	3%

CV, coefficient of variation; EDTA, ethylene diamine tetra-acetate; HDL, high density lipoprotein; LDL, low density lipoprotein; Na-F, sodium fluoride

Table 4.2: Cardiovascular risk factors by tertiles of glomerular filtration rate measured by blood spot iohexol clearance

	All	≤91 ml/min/1.73m ²	92-112 ml/min/1.73m ²	≥113 ml/min/1.73m ²	<i>p</i>
<i>n</i>	106	35	35	36	
GFR (<i>ml/min/1.73m²</i>)	103.1±28.3	74.3±14.8	101.1±5.8	134.1±18.2	
Age (<i>years</i>)	54.3±14.4	59.9±14.3	53.2±13.1	49.7±14.1	0.003†
Female	47%	41%	49%	50%	0.552*
Prior CVD	36%	56%	29%	25%	0.01*
Lipid lowering treatment	43%	64%	34%	31%	0.006*
BP lowering treatment	45%	63%	37%	25%	0.001*
Possible folic acid	35%	53%	26%	25%	0.019*
Current smoker	7%	6%	6%	8%	0.659*
Ex-smoker	42%	50%	34%	40%	0.471*
Systolic BP (<i>mmHg</i>)	129.9±18.6	129.1±17.2	129.8±20.2	130.8±18.7	0.668†
Diastolic BP (<i>mmHg</i>)	78.7±10.4	77.4±10.0	78.2±10.8	80.4±10.4	0.236†
BMI (<i>kg/m²</i>)	26.2 (23.5-29.0)	26.4 (23.0-28.9)	25.8 (23.6-28.9)	26.4 (24.0-29.0)	0.851†
Waist (<i>cm</i>)	91.2±13.0	93.4±13.9	90.8±11.5	89.4±13.6	0.192†
Percentage body fat	29.6±8.6	30.8±9.8	28.3±8.9	29.4±6.8	0.439†
Total-C (<i>mmol/l</i>)	4.4 (3.6-5.1)	4.6 (3.6-5.1)	4.4 (3.6-5.0)	4.3 (3.8-5.1)	0.911†
LDL-C (<i>mmol/l</i>)	2.3 (2.0-3.1)	2.4 (1.7-3.0)	2.5 (2.1-3.2)	2.3 (2.0-3.2)	0.582†
HDL-C (<i>mmol/l</i>)	1.3 (1.0-1.6)	1.3 (1.0-1.6)	1.2 (1.1-1.7)	1.4 (1.0-1.6)	0.913†
Apo b/apo a ₁ ratio	0.59 (0.47-0.73)	0.53 (0.44-0.74)	0.60 (0.53-0.70)	0.57 (0.47-0.73)	0.783†
Triglyceride (<i>mmol/l</i>)	1.12 (0.68-1.91)	1.28 (0.75-2.35)	1.13 (0.88-1.84)	0.97 (0.49-1.56)	0.01†
Fibrinogen (<i>g/l</i>)	3.2±0.6	3.4±0.7	3.1±0.5	3.1±0.6	0.046†
CRP (<i>mg/l</i>)	0.8 (0.7-1.0)	1.0 (0.8-1.2)	0.8 (0.7-0.9)	0.8 (0.7-0.9)	0.497†
Homocysteine (<i>μmol/l</i>)	9.6 (8.3-11.2)	10.6 (8.4-14.3)	9.8 (8.8-11.7)	8.9 (7.9-10.1)	0.001

Data are expressed as mean ± standard deviation, median (range) or percentage

* *p* value for Chi squared test for linear trend

† *p* value for ANOVA test for linear trend (based on geometric mean if variable not normally distributed)

CVD, cardiovascular disease; BP, blood pressure; mmHg, millimeters of mercury; BMI, body mass index;; kg, kilograms; m, meters; cm, centimeters; Total-C, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; Apo, apolipoprotein; CRP, C-reactive protein.

Table 4.3: Summary statistics describing the relationships between GFR measures and blood concentration of lipids

Risk factor	Renal marker	Correlation coefficient	β regression coefficient \pm SE
Log[HDL-C (mmol/l)]	BSIC-GFR	0.167 ($p=0.087$)	0.00040 \pm 0.00041 ($p=0.327$)
Log[HDL-C (mmol/l)]	MDRD-eGFR	0.136 ($p=0.165$)	0.00130 \pm 0.00066 ($p=0.053$)
Log[HDL-C (mmol/l)]	Cyc-eGFR	0.390 ($p<0.001$)	0.00217 \pm 0.00065 ($p=0.001$)
Log[triglyceride (mmol/l)]	BSIC-GFR	-0.247 ($p=0.011$)	-0.00164 \pm 0.00103 ($p=0.115$)
Log[triglyceride (mmol/l)]	MDRD-eGFR	-0.278 ($p=0.004$)	-0.00380 \pm 0.00167 ($p=0.025$)
Log[triglyceride (mmol/l)]	Cyc-eGFR	-0.392 ($p<0.001$)	-0.00468 \pm 0.00168 ($p=0.006$)
Log[apolipoprotein A ₁ (mg/dl)]	BSIC-GFR	0.083 ($p=0.397$)	0.00018 \pm 0.00025 ($p=0.487$)
Log[apolipoprotein A ₁ (mg/dl)]	MDRD-eGFR	0.075 ($p=0.443$)	0.00093 \pm 0.00041 ($p=0.024$)
Log[apolipoprotein A ₁ (mg/dl)]	Cyc-eGFR	0.283 ($p=0.003$)	0.00128 \pm 0.00040 ($p=0.002$)

Correlation coefficients are calculated by Pearson correlation (p for comparison with an r of zero), β regression coefficients are calculated per unit increase in renal variable by linear regression analysis adjusted for age and sex (p for a t-test comparing the β coefficient with zero).
SE, standard error; HDL-C, high density lipoprotein cholesterol; BSIC-GFR, blood spot iohexol clearance GFR; MDRD-eGFR, GFR estimated from the Modification of Diet in Renal Disease formula; Cyc-eGFR, GFR estimated from blood cystatin c concentration.

Table 4.4: Summary statistics describing the relationships between GFR measures and “novel” cardiovascular biomarkers

Risk factor	Renal marker	Correlation coefficient	β regression coefficient \pm SE
Log[CRP (mg/l)]	BSIC-GFR	-0.148 ($p=0.128$)	-0.00152 \pm 0.00153 ($p=0.322$)
Log[CRP (mg/l)]	MDRD-eGFR	-0.140 ($p=0.151$)	-0.00253 \pm 0.00252 ($p=0.317$)
Log[CRP (mg/l)]	Cyc-eGFR	-0.304 ($p=0.002$)	-0.00655 \pm 0.00248 ($p=0.010$)
Fibrinogen (g/l)	BSIC-GFR	-0.141 ($p=0.150$)	-0.00027 \pm 0.00201 ($p=0.894$)
Fibrinogen (g/l)	MDRD-eGFR	-0.378 ($p<0.001$)	-0.00715 \pm 0.00323 ($p=0.029$)
Fibrinogen (g/l)	Cyc-eGFR	-0.404 ($p<0.001$)	-0.00927 \pm 0.00322 ($p=0.005$)
Log[Homocysteine (μ mol/l)]	BSIC-GFR	-0.415 ($p<0.001$)	-0.00175 \pm 0.00037 ($p<0.001$)
Log[Homocysteine (μ mol/l)]	MDRD-eGFR	-0.414 ($p<0.001$)	-0.00329 \pm 0.00059 ($p<0.001$)
Log[Homocysteine (μ mol/l)]	Cyc-eGFR	-0.422 ($p<0.001$)	-0.00332 \pm 0.00062 ($p<0.001$)

Analysis is restricted to the participants without missing data for the variables examined (n=106 for CRP and fibrinogen and CRP and 105 for homocysteine).

Correlation coefficients are calculated by Pearson correlation (p for comparison with an r of zero), β regression coefficients are calculated per unit increase in renal variable by linear regression analysis adjusted for age and sex (p for a t-test comparing the β coefficient with zero).

SE, standard error; CRP, C-reactive protein; BSIC-GFR, blood spot iohexol clearance GFR; MDRD-eGFR, GFR estimated from the Modification of Diet in Renal Disease formula; Cyc-eGFR, GFR estimated from blood cystatin c concentration.

Table 4.5: Summary statistics describing the relationships between measures GFR and markers of body composition

Risk factor	Renal marker	Correlation coefficient	β regression coefficient \pm SE
LogBMI (kg/m ²)]	BSIC-GFR	0.027 ($p=0.785$)	0.00037 \pm 0.00027 ($p=0.173$)
LogBMI (kg/m ²)]	MDRD-eGFR	-0.090 ($p=0.358$)	-0.00014 \pm 0.00044 ($p=1.000$)
LogBMI (kg/m ²)]	Cyc-eGFR	-0.330 ($p=0.001$)	-0.00089 \pm 0.00044 ($p=0.046$)
Waist (cm)	BSIC-GFR	-0.157 ($p=0.113$)	-0.01064 \pm 0.04002 ($p=0.791$)
Waist (cm)	MDRD-eGFR	-0.158 ($p=0.110$)	-0.05089 \pm 0.06565 ($p=0.440$)
Waist (cm)	Cyc-eGFR	-0.507 ($p<0.001$)	-0.20484 \pm 0.06367 ($p=0.002$)
Body fat (%)	BSIC-GFR	-0.016 ($p=0.872$)	0.01531 \pm 0.02438 ($p=0.532$)
Body fat (%)	MDRD-eGFR	-0.307 ($p=0.002$)	-0.04287 \pm 0.39776 ($p=0.284$)
Body fat (%)	Cyc-eGFR	-0.242 ($p=0.014$)	-0.11703 \pm 0.03910 ($p=0.003$)

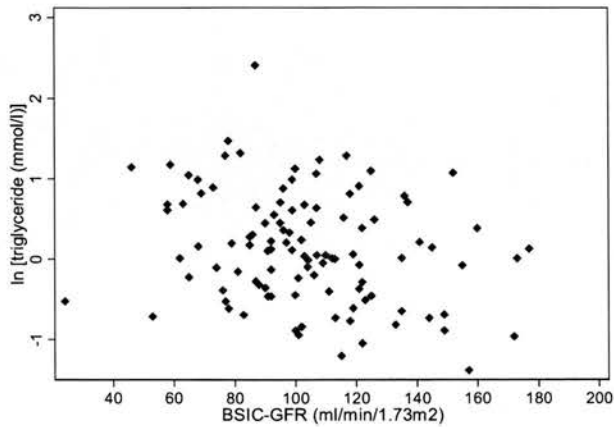
Analysis is restricted to the participants without missing data for the variables examined (n=104 for waist, n=106 for BMI and n=103 for body fat %).

Correlation coefficients are calculated by Pearson correlation (p for comparison with an r of zero); β regression coefficients are calculated per unit increase in renal variable by linear regression analysis adjusted for age and sex (p for a t-test comparing the β coefficient with zero).

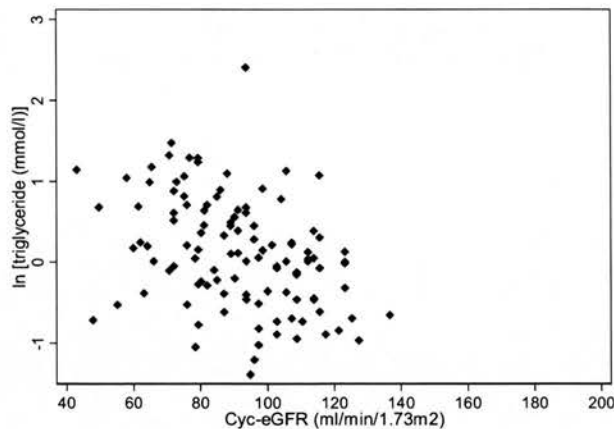
SE, standard error; BMI, body mass index; BSIC-GFR, blood spot iohexol clearance GFR; MDRD-eGFR, GFR estimated from the Modification of Diet in Renal Disease formula; Cyc-eGFR, GFR estimated from blood cystatin c concentration

Figure 4.1: Scatter plots showing the relationship between measures of glomerular filtration rate and Log [blood triglyceride concentration]

a. Blood Spot Iohexol Clearance GFR (BSIC-GFR)



b. GFR estimated from the Modification of Diet in Renal Disease formula (MDRD-eGFR)



c. GFR estimated from blood cystatin c concentration (cyc-eGFR)

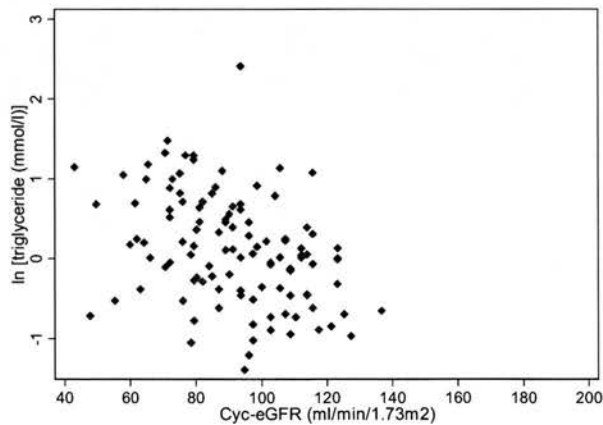
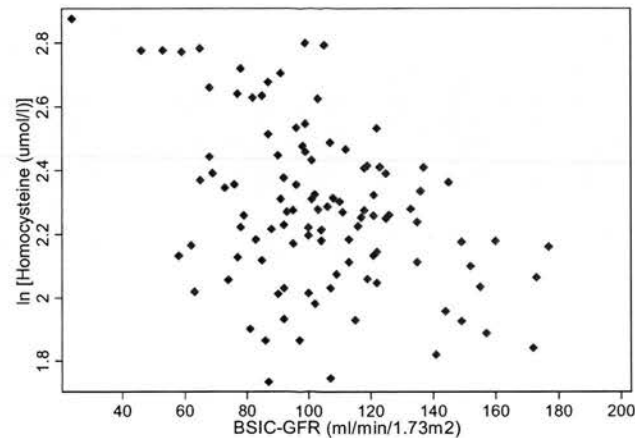
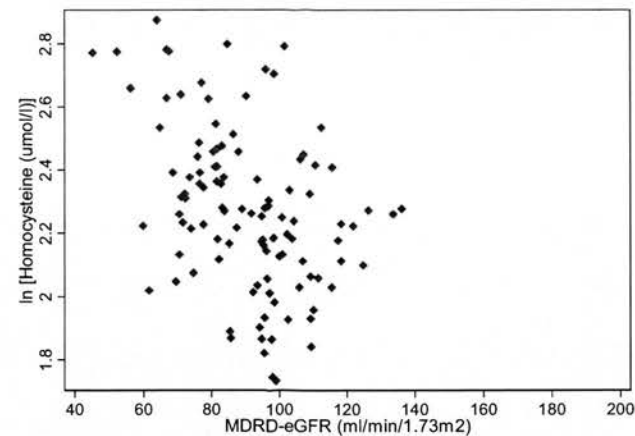


Figure 4.2: Scatter plots showing the relationship between measures of glomerular filtration rate and Log [homocysteine concentration]

a. Blood Spot Iohexol Clearance GFR (BSIC-GFR)



b. GFR estimated from the Modification of Diet in Renal Disease formula (MDRD-eGFR)



c. GFR estimated from blood cystatin c concentration (cyc-eGFR)

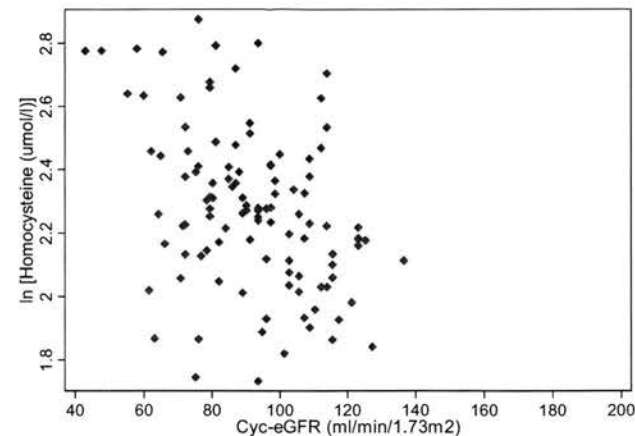
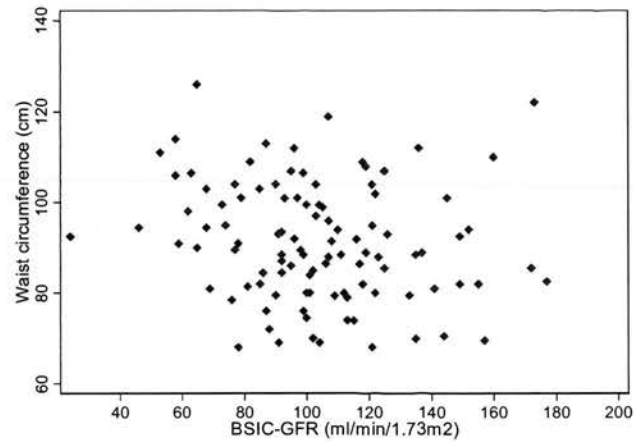
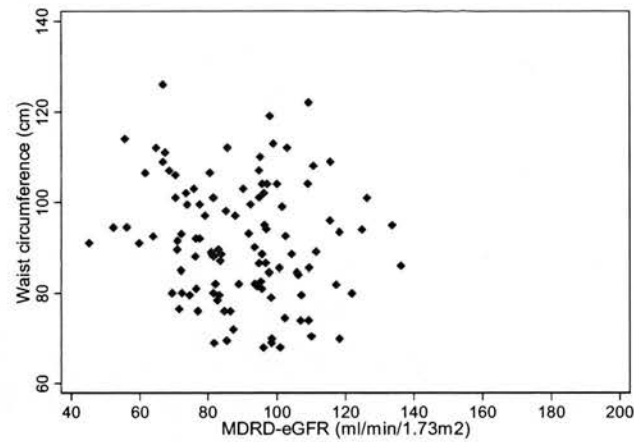


Figure 4.3: Scatter plots showing the relationship between measures of glomerular filtration rate and waist circumference

a. Blood Spot Iohexol Clearance GFR (BSIC-GFR)



b. GFR estimated from the Modification of Diet in Renal Disease formula (MDRD-eGFR)



c. GFR estimated from blood cystatin c concentration (cyc-eGFR)

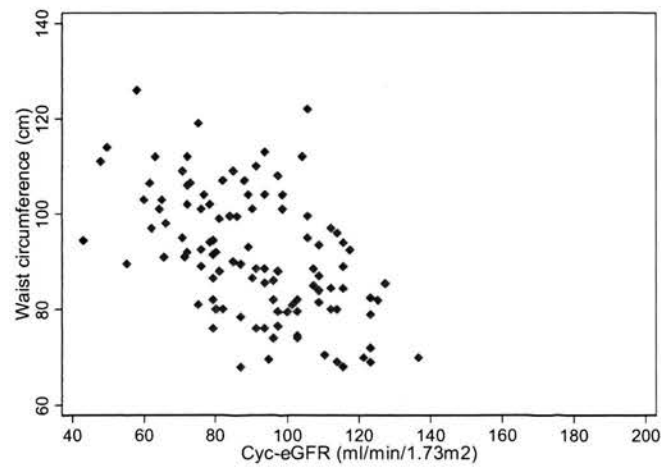
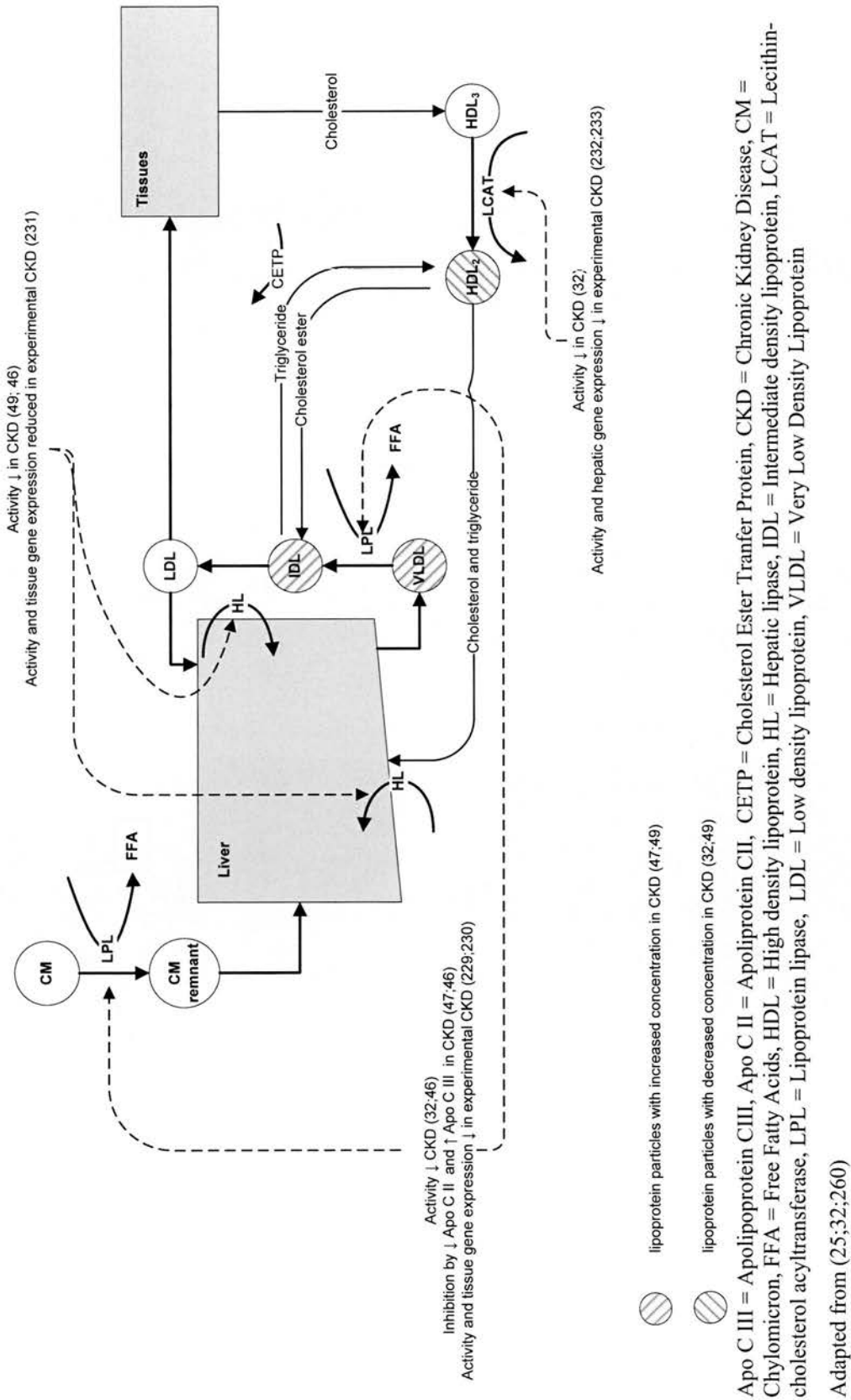


Figure 4.4: Alterations in cholesterol and triglyceride metabolism in human and animal models of chronic kidney disease



Chapter 5: Estimated glomerular filtration rate and the risk of major vascular events and all-cause mortality

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5.1 Aims of the study

Several prospective cohort studies have suggested that mild-to-moderate reductions in GFR may be associated with a modest, but clinically important, increase in the risk of major vascular events (17;18;261;262) and all-cause mortality (263) but previous meta-analyses of such studies (264;265) have been limited by a number of methodological problems.

First, different studies have tended to use different cut points for defining categories of renal function (which make direct between-study comparisons unreliable). Furthermore, estimated GFR is heavily dependent on the laboratory calibration of the creatinine assay (167), and, to a lesser extent, the estimation method used (266), further increasing the likelihood of misclassification between renal function categories when comparing different studies. In addition, studies considering more than two renal function groups typically report relative risks compared with a single reference group assigned the relative risk of 1 with no associated error. While standard practice, this makes it difficult to assess the true nature of dose-response risk relationships.

This quantitative meta-analysis aims to overcome these problems through consideration, not of absolute eGFR levels, but of within-study proportional differences in eGFR, which are less susceptible to biases introduced by laboratory creatinine calibration differences between studies, and by differences in the cut-points used to define renal function groups. Furthermore, by consideration of the number of people and number of events in each group, dose-response risk relationships are constructed that take account

of the information in all risk groups, including the reference groups. Using these methods, this study aims to summarise the risk of death and major vascular events associated with lower eGFR across the published studies, and compare these risks between different types of study.

5.2 Methods

5.2.1 Search strategy and inclusion criteria

Studies assessing the relationship between eGFR (estimated using either the MDRD formula (114;116) or the Cockcroft-Gault formula (115)) and major cardiovascular events (MVE; see below) or all cause mortality were identified from MEDLINE by searching PubMed (1966 to 1st August 2006), using a combination of terms relating to cardiovascular disease or death, creatinine or eGFR, and cohort studies (Table 5.1). The MEDLINE search was complemented by reviewing the reference lists of subsequently retrieved papers, and, where necessary, through contact with study authors. The pre-specified criteria for inclusion into the meta-analysis were studies;

- assessing all-cause mortality or major vascular events (defined as myocardial infarction; unstable angina; stroke; transient ischaemic attack; coronary or non coronary revascularisation; or cardiovascular, cardiac or coronary death),
- of at least 500 individuals,
- involving at least 1 year planned follow-up, and
- which reported risk associations that were adjusted for at least age and sex.

Studies were excluded if they only analyzed eGFR as a continuous variable or if the study was conducted solely among individuals with existing chronic kidney disease or other serious non-cardiovascular disease likely to be related to kidney dysfunction (e.g. chronic liver disease).

5.2.2 Data extraction and definition of terms

The following data were extracted from each study;

- details of the study population,
- mean follow-up,
- the type and total number of outcomes,
- details of each category of eGFR (the cut points used, the mean eGFR for the group and the number of individuals and outcomes in the group),
- the most fully adjusted relative risk estimates and confidence intervals associated with each comparison group, and
- the confounders adjusted for in the analysis.

If a study reported results for both Cockcroft Gault and MDRD eGFR, the results using the MDRD formula were extracted. Studies were considered to be a 'prospective cohort study' if the baseline data were prospectively collected by study personnel but not as part of a randomized controlled trial. Studies which analysed registry data or retrospectively extracted data from hospital medical records were considered 'routine data' sources. In the case of duplicate reports from the same study population, the first published paper was used unless the later report contained additional events.

5.2.3 Statistical methods

The primary analyses involved estimating the relative risk of MVE and all-cause mortality associated with a 30% lower eGFR. First, the proportional difference in eGFR

for each comparison group compared to the reference group was identified and then, for each study, the risk associated with a 30% lower eGFR was calculated.

5.2.3.1 Calculating the proportional difference in GFR for each comparison group

Where the mean eGFR in each group was not provided, it was estimated either through knowledge of the mean and standard deviation of the overall distribution, or else through the proportions of the population falling within each group (see section 5.2.3.2 below) assuming, unless stated otherwise in the publication, that eGFR followed a normal distribution since this was the distribution observed in 20536 people at high-risk of vascular disease who were enrolled into the Heart Protection Study (Figure 5.1). In one study, a log-normal distribution was assumed rather than a normal distribution because the authors explicitly stated that the distribution was positively skewed (the mean values in this study therefore represent geometric means). This approach was validated using data from the studies that did report mean eGFR in each group (Figure 5.2).

5.2.3.2 Estimating the mean eGFR level in groups defined by ‘cutoff’ values

If X has a normal distribution with mean μ and standard deviation σ , then it follows that

$Z = (X - \mu) / \sigma$ has a normal distribution with mean 0 and standard deviation 1. Now

let X' be the same as X but constrained to lie between two cutoff values a and b . The

expected value of X' is equal to $E(X | a < X < b) = \mu + \sigma E(Z | \frac{a-\mu}{\sigma} < Z < \frac{b-\mu}{\sigma})$. The

probability density function of Z conditional on Z taking a value between $\frac{a-\mu}{\sigma}$ and $\frac{b-\mu}{\sigma}$ is:

$$\Pr\left(Z < z \mid \frac{(a-\mu)}{\sigma} < Z < \frac{(b-\mu)}{\sigma}\right) = \frac{1}{\Phi\left(\frac{(b-\mu)}{\sigma}\right) - \Phi\left(\frac{(a-\mu)}{\sigma}\right)} \int_{\frac{(a-\mu)}{\sigma}}^z \phi_w dw$$

where ϕ is the standard normal density function and Φ is the standard normal cumulative distribution function. Therefore, the expected value of X' is equal to:

$$\begin{aligned} & \mu + \frac{\sigma}{\Phi\left(\frac{(b-\mu)}{\sigma}\right) - \Phi\left(\frac{(a-\mu)}{\sigma}\right)} \int_{\frac{(a-\mu)}{\sigma}}^{\frac{(b-\mu)}{\sigma}} w \phi_w dw \\ &= \mu + \frac{\sigma}{\Phi\left(\frac{(b-\mu)}{\sigma}\right) - \Phi\left(\frac{(a-\mu)}{\sigma}\right)} \cdot \frac{1}{\sqrt{2\pi}} \left(e^{-\frac{1}{2}\left(\frac{(a-\mu)}{\sigma}\right)^2} - e^{-\frac{1}{2}\left(\frac{(b-\mu)}{\sigma}\right)^2} \right) \end{aligned} \quad (1)$$

However, in studies in which the overall mean and standard deviation were not stated, these were first estimated from the known proportions of the population falling within each exposure group. For instance, if a study of N participants reported three exposure groups: $\text{eGFR} < a$; $\geq a, < b$ and $\geq b$ ml/min/1.73m²; with n_1 , n_2 and n_3 participants falling, respectively, into each category, then, under the normality assumption:

$$\frac{a - \hat{\mu}}{\hat{\sigma}} = \Phi^{-1}(p_1)$$

and

$$\frac{b - \hat{\mu}}{\hat{\sigma}} = \Phi^{-1}(p_2)$$

where $p_1 = n_1/N$ and $p_2 = (n_1 + n_2)/N$, and so:

$$\hat{\sigma} = \frac{b - a}{\Phi^{-1}(p_2) - \Phi^{-1}(p_1)} \quad (2)$$

and

$$\hat{\mu} = a - \hat{\sigma} \cdot \Phi^{-1}(p_1) \quad (3)$$

For studies in which more than 3 exposure groups were used however (and hence any two cutoff values could potentially be used to calculate the mean and standard deviation of the overall distribution), the mean eGFR in each exposure group was calculated from estimates of μ and σ derived from the nearest two cutoff levels (i.e. either the cutoff levels actually defining the group or, if the group is one of the tails of the distribution, from the two nearest cutoff levels).

5.2.3.3 Calculating summary risk estimates for each study

For studies in which participants were separated into one of only two groups, the reported relative risk (and its 95% confidence interval) was simply rescaled by assuming a log-linear relationship between log eGFR and risk. For example, if a study reported a relative risk of 1.5 when comparing individuals with mean eGFR 46 ml/min/1.73m² with individuals with mean eGFR 85 ml/min/1.73m² (i.e. a *relative* difference in mean eGFR of 46/85 = 0.54), the rescaled relative risk for a 30% lower eGFR would be 1.5 x 0.806 = 1.21 (since 0.54 / 0.67 = 0.806). [Formally, the rescaled relative risk is obtained from the published relative risk (RR) through the equation $\exp(\ln(0.67) \times \ln(RR) / \ln(a/b))$, where b is the mean exposure level in the reference group and a is the mean exposure level in the single comparison group].

For studies in which more than two groups were considered, the relative risk was derived from the slope of a weighted linear regression through the reported (log) relative risk estimates (including through the relative risk of 1 corresponding to the reference

group) against log eGFR. Weights for the groups (including for the reference group) were obtained by reconstructing the approximate (uncorrelated) standard errors of the crude 'floating absolute risks' (267). Unfortunately, in all of the studies, relative risks were reported relative to a single reference group (assigned the relative risk of 1) with *no associated error*. Not only does this make the confidence intervals around the relative risk estimates correlated, but, more importantly, it also makes it impossible to determine the amount of 'statistical information' in the reference group (and hence impossible to determine the appropriate weighting for the reference group). Logistic regression was therefore used to 're-analyze' each study, relating the exposure group to the number of subjects and events observed in each group. This provided a variance-covariance matrix of the estimated (crude) log odds ratios which was used to calculate approximate 'floated' standard errors for the log odds ratio in *each* group (including the reference group) (267). While the odds ratios obtained from these analyses would be expected to differ from the adjusted relative risks actually published, their floated standard errors should be close to the true floated standard errors because they are chiefly determined by the number of subjects and events in each group, and should therefore be relatively unaffected by adjustment for confounders. The risk of MVE or all-cause mortality associated with a 30% lower eGFR was then estimated from the slope provided by the weighted linear regression of the *published* log relative risks on the (log) mean exposure levels (with weights equal to the reciprocal of the square of the floated standard errors, i.e. inverse variance weighted). However, since these weights were known exactly (and not just relatively), the standard error of the slope needed to be corrected by dividing it by the square root of the mean squared error (268).

5.2.3.4 Assessing heterogeneity between studies

Given the summary log relative risk b_i (and its variance v_i) for each study (see above), heterogeneity between the different studies was assessed by calculating $X = \Sigma(w_i b_i^2) - \Sigma(w_i b_i)^2 / \Sigma w_i$ (where $w_i = 1/v_i$), and testing this against a chi-squared distribution with $k-1$ degrees of freedom (where k is the number of studies). This was done separately within particular types of study (e.g. prospective cohort studies, randomised controlled trials), and also separately in populations with and without prior vascular disease. The 'pooled' log relative risk across different studies was calculated by $\Sigma w_i b_i / \Sigma w_i$ (with variance equal to $1 / \Sigma w_i$).

5.2.3.5 Regression dilution bias

With the exception of one study (261), in which analyses were performed using time-dependent covariate techniques, all results presented in this paper are derived purely from analyses of baseline eGFR measures. The potential effects of regression dilution bias (i.e., the tendency for baseline measures to result in underestimation of associations with long-term 'usual values' (269)) on the findings of the meta-analysis were considered through examination of repeated eGFR measurements taken an average of 4.5 years apart in 7697 patients allocated placebo in the MRC/BHF Heart Protection Study (270).

5.3 Results

5.3.1 *Included studies*

Figure 5.3 summarizes the search retrieval process. Out of 9583 abstracts reviewed, 124 papers were retrieved for further examination, of which 43 met the inclusion criteria, with 5 more being identified from the reference lists. Contained within these 48 manuscripts was information relating to 53 different studies. Mean eGFR in the different risk groups was presented (or else sufficient information was provided to allow its estimation) for 48 of these studies, including 12 prospective cohort studies, 19 randomised controlled trials and 17 studies from 'routine' sources (Table 5.2). In studies where 'pooled' analyses had been performed in the original papers (18;271;272), authors were contacted to provide study-specific results, while in the largest study (263), where age specific analyses had been performed, the authors were contacted to provide results separately for people with and without prior vascular disease, both age-specifically, and overall for all ages.

5.3.2 *eGFR and the risk of major vascular events*

Twenty six studies (12 cohort studies, 11 trials and 3 routine data sources) comprising a total of 1 372 820 individuals assessed the relationship between eGFR and major vascular events. Among these studies, 842 762 individuals (61% of all participants) were included in the reference groups, in which the weighted mean (SD) eGFR across the studies was 83 ml/min/1.73m² (12.8 ml/min/1.73m²). Figure 5.4a shows the overall dose-response relationship between MVE risk and proportional differences in eGFR

from this reference level; Figure 5.5 shows study-specific estimates of the relative risk associated with a 30% lower eGFR, grouped by study design and by history of prior vascular disease. A strong, graded, approximately log-linear relationship was observed across the different studies, with lower eGFR levels consistently related to higher MVE risk, at least down to eGFR levels of about 25-30 ml/min/1.73m² (i.e., about 60-70% lower than average reference levels) (Figure 5.4a). A 30% lower eGFR level (e.g., from ~80 to 55 ml/min/1.73m²) was associated with a 20-30% increased risk of MVE, irrespective of study design, or of history of prior vascular disease (Figure 5.5). However, substantial heterogeneity was observed within the prospective cohort studies that could not be accounted for by separation according to prior disease. The two studies from routine data sources in people with prior vascular disease also gave apparently discordant results. There was no significant heterogeneity between the findings of the randomised controlled trials however (combined relative risk among studies of patients with prior disease: 1.19 [95% CI 1.16 to 1.21] for a 30% lower eGFR).

5.3.3 eGFR and risk of all-cause mortality

Forty one studies (9 cohort studies, 15 trials and 17 routine data sources) comprising a total of 4 061 003 individuals assessed the relationship between eGFR and all-cause mortality. Among these studies, 2 888 629 individuals (71% of all participants) were included in the reference groups, in which the weighted mean (SD) eGFR across the studies was 84 ml/min/1.73² (11.2 ml/min/1.73m²). Figure 5.4b shows the overall dose-response risk relationship with proportional differences in eGFR and Figure 5.6 shows the study-specific relative risks associated with a 30% lower eGFR, by study design and

by history of prior vascular disease. As for MVE, a strong, graded relationship was observed between lower eGFR levels and progressively higher all-cause mortality risks (Figure 5.4b), with each 30% lower eGFR level being associated with, on average, about a 20-30% increase in all-cause mortality (Figure 5.6). Within both the prospective cohort studies and the routine data sources, there was substantial heterogeneity between the findings of the individual studies, which could not be accounted for by prior vascular disease. However, there was again no significant heterogeneity between the findings of the randomised controlled trials (combined relative risk among studies of patients with prior disease: 1.23 [95% CI 1.20 to 1.26] for a 30% lower eGFR). The absolute relevance of reductions in eGFR to all-cause mortality risk was some 3-4 times greater among populations with prior CVD or other 'high-risk' populations than among populations without prior CVD (Figure 5.7).

5.3.4 eGFR and all-cause mortality risk by age

In one very large study of eGFR and all-cause mortality (the Veterans Affairs Study (263); comprising 2.6 million participants [64% of all the individuals followed for all-cause mortality] and 218 000 deaths [69% of all the deaths]), age-specific results were made available by the authors. The relative risk of all-cause mortality at different eGFR levels, among people with and without a history of prior vascular disease, and separately by age group is shown in figures 5.8 and 5.9: The relative importance of eGFR levels to all-cause mortality risk decreased as age increased (Figure 5.8). However, since older people and people with prior vascular disease were at substantially higher absolute all-cause mortality risk, the absolute relevance of eGFR levels to risk increased markedly

with age and with prior vascular disease (Figure 5.9). In most people (certainly those aged ≥ 55 years), differences in eGFR levels between about 60 and 90 ml/min/1.73m² corresponded to no material increase in all-cause mortality risk. Therefore the ‘summary’ relative risks shown in figure 5.6 across the entire range of eGFR levels may overestimate the true relative risks observed at higher eGFR levels and underestimate the true relative risks at lower levels.

5.3.5 Assessment of the potential effects of regression dilution bias

To investigate the extent that the present use of baseline measurements of eGFR might result in regression dilution bias, i.e., underestimation of the relation between long-term ‘usual’ eGFR levels and risk, repeated eGFR measurements taken an average of 4.5 years apart in 7697 patients allocated placebo in the MRC/BHF Heart Protection Study (270) were examined (Figure 5.10). Mean eGFR (estimated using the MDRD equation (114)) was 74 ml/min/1.73m² at baseline and 67 ml/min/1.73m² upon re-measurement. As expected, patients with initial eGFR levels above the mean had follow-up levels somewhat nearer to the mean, on average. However, patients with baseline eGFR levels substantially below the mean had similar (slightly lower) levels upon re-measurement indicating that their eGFR levels decreased, on average, at a faster rate than that observed in the population as a whole. The correlation between eGFR levels at baseline and follow-up was 0.68.

5.4 Discussion

5.4.1 Overall findings and potential sources of bias

This quantitative meta-analysis of cohort studies demonstrates an independent, graded inverse relationship between proportional differences in baseline eGFR and risk of both all cause mortality and major vascular events. A 30% lower eGFR was associated with approximately a 20-30% increase in the risk of both outcomes (Figures 5.5 and 5.6). However, this may represent a distortion of the true strength of the relationship between GFR and these outcomes for several reasons.

First, in order to summarise the increase in risk of death and cardiovascular disease associated with lower eGFR levels between the studies, it was necessary to ‘scale’ the reported risk estimates to a standard proportional difference in GFR assuming a log-linear relationship. Examination of the individual dose response relationships in figure 5.4 suggested that, across most of the range of GFR differences, this was a reasonable assumption. However, for all cause mortality, small reductions in eGFR, of around 20% or less (e.g., from ~85 ml/min to ~70 ml/min), were not associated with any increase in risk of death. This observation was largely attributable to 2 large ‘routine data’ sources (261;263), which together contributed 85% of the deaths, in which lower eGFR was not associated with increased risk of death until eGFR was below 60 ml/min/1.73m². Therefore, the summary estimates shown in figure 5.6 may well underestimate the strength of the true relationship between lower GFR and death among those with eGFR <60 ml/min/1.73m².

Second, because most of the studies (all but one) used baseline eGFR in analyses, true dose-response risk-relationships may have been distorted because of regression dilution bias (269). To investigate this potential source of bias, GFR estimates over a 4-5 year period were examined among 7697 individuals allocated placebo in the MRC/BHF Heart Protection Study (270). Among those with an eGFR below the population mean (representative of most of the comparison groups in this meta-analysis), the repeat measurements were lower than those taken at baseline (Figure 5.10). This difference in eGFR between baseline and follow up is likely to represent a combination of progression of renal disease (resulting in the lower eGFR at follow-up) and regression to the mean (resulting in a higher eGFR at follow-up). The individual contribution of these two factors, however, is uncertain, and therefore it is difficult to quantitatively assess the effect that regression dilution bias may have had in the current context, although these results might underestimate the true strength of the relationship between usual eGFR and the risk of these outcomes.

Third, since factors other than GFR affect plasma creatinine concentration (diet, muscle mass and tubular creatinine secretion), for an individual the GFR estimated from serum creatinine concentration can be widely discrepant from directly measured GFR, especially among individuals with preserved GFR (≥ 60 ml/min/1.73m²) (20;21), leading to misclassification of individuals between the groups. In a group of 2095 adults referred for assessment of GFR by clearance chromium-51 ethylene diamine tetra-acetate (51-Cr-EDTA) (21), only 71% were correctly classified into K / DOQI CKD stage (≥ 90 , 60-89, 30-59, 15-29, <15 ml/min/1.73m²) by the MDRD formula (16). This degree of

misclassification might lead to substantial underestimation of the risk-relationships between true GFR and risk of death and cardiovascular events when eGFR is used (170). Since 'true' GFR as not measured in any of these cohort studies the degree to which the use of estimated GFR has biased these results cannot be determined. However, we aimed to explore how the method used to estimate GFR might affect these associations by identifying studies which assessed the relationship between eGFR or blood creatinine concentration, *and* blood cystatin c concentration and all cause mortality. Searching MEDLINE 1966 -1 April 2007 using the term 'cystatin', identified 5 studies assessing blood cystatin concentration and death, of which 3 provided age and sex adjusted results for both cystatin c and creatinine based GFR measures (Table 5.3). In each study, blood cystatin c concentration was more strongly associated with an increased risk of death than blood creatinine concentration or eGFR (Table 5.3). Possible explanations for these findings include bias introduced by non-renal influences on blood cystatin c concentration or a direct biological effect of cystatin c itself: However, it is possible that the stronger relationship seen between blood cystatin c concentration and risk of death is the result of a stronger relationship between blood cystatin c concentration and 'true' GFR.

5.4.1.1 Impact of age on the relationship between eGFR and all cause mortality

The large Veteran's Affairs study observed a progressive weakening of the relative increase in risk of death associated with lower eGFR as age increases (Figure 5.8). This is consistent with a recently published analysis of a cohort of adults in whom creatinine had been routinely measured, in which the relative risk of death associated with each K /

DOQI CKD stage (16) among those aged 20-44 years was approximately 10 times that observed among those aged over 85 years (273), and is in keeping with the effect of age on the increased risk of cardiovascular death associated with ESKD, where young individuals have an approximated 100 fold increase risk compared to a roughly 10 fold increase in the elderly (274). However, this greater relative risk of death associated with lower eGFR at younger ages is due to the very low background risk in these individuals, and the absolute increase in risk of death associated with lower eGFR is substantially greater at older ages (Figure 5.9 and (273)).

5.4.2 Potential sources of heterogeneity

After taking into account proportional differences in eGFR at baseline, significant heterogeneity was observed within the individual prospective cohort studies and also within the routine data sources, which was not explained by the presence or absence of prior cardiovascular disease. This heterogeneity could be the result of methodological differences between the studies or differences in the study populations. Individual patient data would be needed to properly explore the reasons for the between study heterogeneity, although one methodological difference is worth mentioning here. In the Kaiser-Permanente study (261), time-dependent techniques (rather than baseline eGFR) were used to model the relationship between eGFR and risk which might explain the somewhat stronger associations observed in this study (Figures 5.5 and 5.6).

5.4.3 Possible explanations for the observed associations

The potential explanations for the increased risk of cardiovascular events and death associated with lower eGFR include residual confounding, reverse causality or a causal relationship. First, although almost all of the prospective cohort studies and many of the other studies reported risk estimates adjusted not only for age and sex but also for cholesterol, blood pressure, diabetes and smoking, we cannot discount the possibility that residual confounding might remain. However, since the mechanisms by which lower GFR might result in increased risk of cardiovascular disease would be expected to include the development of hypertension and dyslipidaemia, it could equally be argued that adjustment for these factors might attenuate a real relationship between GFR and cardiovascular disease (275). Individual patient data meta-analysis could be used to explore the effect of adjustment for such ‘mediating’ factors on the putative causal pathway and other confounding factors. Second, cardiovascular disease might cause reduced GFR (reverse causality) as a result of generalised atherosclerosis affecting the coronary and renal arteries, nephrotoxic medication use or low output heart failure causing reduced renal perfusion. While the observation that inverse risk-relationships with eGFR were preserved in populations without known prior vascular disease might suggest this to be unlikely, individual patient data meta-analysis of long term studies examining early and late events separately would again be needed to properly explore this possibility. Third, lower GFR might be a causal risk factor for cardiovascular disease (23;276;277). Lower GFR is associated with traditional cardiovascular risk factors such as increased blood pressure (278) and pro-atherogenic blood lipid profile

(5;32) along with non-traditional cardiovascular risk factors such as raised inflammatory markers (59), increased markers of platelet activation (54), higher blood homocysteine concentration (279) and increased oxidative stress (63). However, in this meta-analysis, lower eGFR was associated with a similar increase in the risk of both cardiovascular disease and all cause mortality, suggesting that lower eGFR may also be associated with non-vascular mortality.

5.4.4 Limitations of the study

A number of limitations of this study should be considered. First, a substantial number of the routine data sources (280-290) and four of the randomized controlled trials (291-294) assessing all cause mortality in populations with prior vascular disease included individuals with an acute illness at baseline. Exclusion of these studies, however, did not materially alter the results: A 30% lower eGFR was associated with a 24% increase in the risk of death in both randomised controlled trials and routine data sources after excluding these studies, compared to a 23% increase when all studies were included.

Second, this meta-analysis relied on published risk estimates from whole populations. We were, therefore, unable to explore the impact of adjusting for various confounders, to analyse subgroups separately, or to examine the relationship between eGFR and separate cardiovascular events or cause specific mortality. In addition, since the mean eGFR in the reported reference groups was around 84 ml/min/1.73m² this study cannot make any comment on the impact of eGFR at higher eGFR values. Individual patient data meta-analysis of these studies could potentially overcome these problems. However,

since all these studies estimated GFR from serum creatinine concentration, which is only weakly related to GFR among those with $\text{GFR} > 60\text{ml/min/1.73m}^2$ (173), such analyses may not reliably assess true risk-relationships across the range of GFR values.

5.5 Summary of key findings

- A strong, graded, inverse relationship between lower eGFR levels and increased risks of death and major vascular events was consistently observed in cohort studies, randomized controlled trials and studies of routine data sources among people with and without prior cardiovascular disease.
- A 30% lower eGFR was associated with approximately a 20-30% increase in the risk of both outcomes, although there was significant heterogeneity between the individual prospective cohort studies and routine data sources.
- These estimates may distort the true relationship between usual GFR and risk of cardiovascular disease and death as a result of regression dilution bias and misclassification due to inaccuracies of eGFR.
- The relative importance of eGFR levels to all-cause mortality risk decreased as age increased, but the absolute relevance of eGFR levels to risk was greatest at older ages and among those with prior vascular disease.
- Individual patient data meta-analysis is now needed to allow analysis of specific subgroups, examination of individual outcomes, exploration of the effect of adjustment for confounders and full assessment of the dose response relationship although the large discrepancies between estimated and true GFR may still lead to an underestimation of the true relationship between GFR and cardiovascular disease and death.

Chapter 5

Tables and figures

Table 5.1: PubMed search conducted on 1st August 2006

Step		Citations
1	“cardiovascular diseases/epidemiology”[MeSH Terms] OR “heart diseases/epidemiology”[MeSH Terms] OR “coronary disease/epidemiology”[MeSH Terms] OR “myocardial infarction/epidemiology”[MeSH Terms] OR “cerebrovascular disorders/epidemiology”[MeSH Terms] OR “death/epidemiology”[MeSH Terms] OR “vascular diseases/epidemiology”[MeSH Terms] OR “cardiovascular abnormalities/epidemiology”[MeSH Terms]	130071
2	“renal failure” OR “renal impairment” OR “renal disease” OR “kidney disease” OR “chronic kidney failure” OR “chronic kidney insufficiency” OR “glomerular filtration” OR “glomerular filtration rate” OR “kidney function test” OR “kidney function tests” OR “kidney function” OR “kidney function creatinine clearance” OR “kidney function serum creatinine” OR “kidney clearance” OR “kidney clearance rates” OR “kidney clearance tests” OR “kidney clearances”	148049
3	#1 AND #2	4175
4	“cardiovascular diseases “ OR “heart diseases” OR “coronary disease “ OR “myocardial infarction “ OR “cerebrovascular disorders “ OR “death “ OR “vascular diseases “ OR “cardiovascular abnormalities “	685383
5	“renal failure” OR “renal impairment” OR “renal disease” OR “kidney disease” OR “chronic kidney failure” OR “chronic kidney insufficiency” OR “glomerular filtration” OR “glomerular filtration rate” OR “kidney function test” OR “kidney function tests” OR “kidney function” OR “kidney function creatinine clearance” OR “kidney function serum creatinine” OR “kidney clearance” OR “kidney clearance rates” OR “kidney clearance tests” OR “kidney clearances” OR “creatinine/blood” OR “kidney/abnormalities” OR “kidney/epidemiology” OR “kidney/physiology” OR “kidney/physiopathology”	184883
6	“prognosis” OR “risk factors” OR “survival analysis” OR “comparative study” OR “prospective studies” OR “cause of death” OR “cohort study” OR “epidemiology”	2557514
7	#4 AND #5 AND #6	7627
8	#3 NOT #7	1968
9	#7 NOT #3	5420
10	#3 AND #7	2207
11	#8 OR #9 OR #10	9583

Table 5.2: Characteristics of eligible a) prospective cohort studies, b) randomized controlled trials, and c) routine data sources

a) Prospective cohort studies

Study	n	Population	Age <i>mean</i>	Men %	CVD %	FU <i>mean</i>	Outcomes (n)	Comparison groups <i>ml/min or ml/min/1.73m²</i>	Adjustments ¹
Framingham, 2004 (18)	5498	28-62 yrs, USA	52	45	0	9.3	MVE (463), Death (623)	MDRD (<60 vs ≥60)	BLDSR
ARIC, 2004 (18)	12904	45-64 yrs, USA	54	45	0	9.4	MVE (1133), Death (904)	MDRD (<60 vs ≥60)	BLDSR
CHS, 2004 (18)	4201	>65yrs, USA	73	39	0	7.9	MVE (1000), Death (1162)	MDRD (<60 vs ≥60)	BLDSR
NHS, 2004 (295) ²	730	Female nurses, USA	60	0	0	8 ^{††}	MVE [‡] (244)	CG (75-89, 60-74, <60 vs ≥90)	BLDSWR
Rotterdam, 2005 (19)	4484	>55 yrs, Netherlands	70	36	0 ^{‡‡}	8.6	MVE ^{‡‡} (218)	MDRD (quartiles 1, 2, 3 vs 4)	BLVDSW
NIPPON DATA 90, 2006 (296)	7316	>30yrs, Japan	52	33	0	9.6	MVE (183), Death (655)	MDRD (<60 vs ≥60)	BLDSW
JPHC, 2006 (297)	91432	Age 40-69, Japan	58	33	0	10.1	MVE (1994), Death (7070)	MDRD (90-99, 80-89, 70-79, 60-69, <60 vs ≥100)	BLDSW
MONICA Augsburg, 2006 (298)	7534	25-74 yrs, Germany	58	51	5	12.5	MVE (587), Death (1330)	MDRD (15-59 vs ≥60)	BLVDSW
BMES, 2005 (299)	3074	49-97 yrs, Australia	66	43	16	8.2	MVE [‡] (224)	CG (quintile 1 vs 2-5)	BLVDSW
Hoy, 2001 (300)	794	>18 yrs, Aboriginal Australian	33	52		5.8	MVE (26), Death (65)	CG (80-89, 60-79, <60 vs >100)	age, sex only
NHANES II, 2002 (17)	6354	30-74 yrs, USA	49	47	6	13	MVE (797), Death (1240)	MDRD (70-89, <70 vs ≥90)	BLVDSWR
WHCP, 2005 (301)	9939	Hypertension, USA	52	62	11	9.6	MVE (573), Death (1084)	CG (60-74, 45-59, <45 vs ≥75)	BLVDSWR
Framingham, 2004 (272)	741	Prior CVD, USA	68	54	100	6	MVE (240), Death (335)	MDRD (<60 vs ≥60)	BLVDSR
ARIC, 2004 (272)	2102	Prior CVD, USA	56	48	100	5.9	MVE (577), Death (421)	MDRD (<60 vs ≥60)	BLVDSR
CHS, 2004 (272)	1415	>65 yrs, Prior CVD, USA	74	54	100	8.1	MVE (710), Death (718)	MDRD (<60 vs ≥60)	BLVDSR

See page 162 for notes and abbreviations

b) Randomized controlled trials

Study	n	Population	Age mean	Men %	CVD %	FU mean	Outcomes (n)	Comparison groups ml/min or ml/min/1.73m ²	Adjustments ¹
ALLHAT, 2006 (262)	31897	>55 yrs, Hypertension	66	54	26 [‡]	6 ^{††}	MVE (8537)	MDRD (60-89, <60 vs ≥90)	BLDSWR
WOSCOPS, 2004 (271)	6588	High risk of CVD	55	100	5 [‡]	4.9	MVE (514), Death (240)	CG (60-89, <60 vs ≥90)	BLVDS
CARE, 2004 (271)	4148	Previous MI	59	86	100	5 [†]	MVE (592), Death (376)	CG (60-89, <60 vs ≥90)	BLVDS
LIPID, 2004 (271)	8989	Previous MI	62	83	100	6.1	MVE (1555), Death (1127)	CG (60-89, <60 vs ≥90)	BLVDS
TAXUS-IV, 2005 (302)	1300	Stable CAD	62	72	100	5 ^{††}	Death (26)	CG (≤60 vs >60)	BLVDS
BIP, 2006 (303)	6685	Stable CAD	60	78	100	8 ^{††}	MVE [‡] (287)	CG (≤60 vs >60)	BLVDSW
PEACE, 2006 (304)	8280	Stable CAD	64	82	100	4.8 [†]	MVE (696), Death (633)	MDRD (60-74, 45-59, <45 vs ≥75)	BVD
HERS, 2001 (305)	2761	Women with CAD	67	0	100	4.1	MVE (682)	CG (<40, 40-60 vs >60)	BLVDSWR
V A-HIT, 2004 (306)	2505	Men with CAD	64	100	100	5.1 [†]	MVE [‡] (491)	CG (≤75 vs >75)	BLVDSW
TRACE, 2002 (291) ³	6252	Acute MI	68	68	100	6 ^{††}	Death (2787)	CG (71-85, 56-70, 41-55, <40 vs >85)	BVDS
Stent-PAMI, 2003 (293) ³	847	Acute MI	60	75	100	1 ^{††}	Death (38)	CG (≤75 vs >75)	V ⁴
VALLIANT, 2004 (292) ³	14527	Acute MI	65	69	100	2.1 [†]	MVE (5285), Death (3304)	MDRD (60-74, 45-59, <45 vs ≥75)	BLVDSR
SAVE, 2004 (294) ³	2183	Acute MI + LVEF <40%	59	82	100	3.5	MVE (784), Death (492)	MDRD (60-74, 45-59, <45 vs ≥75)	BVDW
SOLVED-P, 2000 (307)	3673	LVEF ≤35%	59	89	100	3.1	Death (564)	CG (≥60 vs <60)	BVD
SOLVED-T, 2000 (307)	2161	HF + LVEF ≤35%	61	81	100	3.45	Death (820)	CG (≥60 vs <60)	BVD
PRIME II, 2000 (308)	1702	HF + LVEF ≤35%	65	80	100	0.75	Death (432)	MDRD (59-76, 44-58, <44 vs >76)	BV ⁴
DiG, 2004 (309)	6800	HF + LVEF <40%	64	77	100	3.1	Death (2375)	MDRD (30-60, <30 vs >60)	BVDWR
MADIT-II, 2006 (310)	1223	CAD + LVEF <30%		84	100	1.7	Death (216)	MDRD (<60 vs ≥60)	VDSW
CHARM, 2006 (311)	2680	HF	65	67	100	2.9 [†]	MVE (950), Death (625)	MDRD (75-89, 60-74, 45-59, <45 vs ≥90)	BVDSR

See page 162 for notes and abbreviations

c) Routine data sources

Study	n	Population	Age mean	Men %	CVD %	FU mean	Outcomes (n)	Comparison groups ml/min or ml/min/1.73m ²	Adjustments ¹
Go, 2004 (261)	1120295	Kaiser Permanente	52	45	6 [†]	2.8 [†]	MVE (139011), Death (51424)	MDRD (45-59, 30-44, 15-29, <15 vs ≥60)	BLVD
O'Hare, 2006 (263)	2598548	Veterans Affairs	64	95	36 [†]	3.2	Death (218246)	MDRD (50-59, 40-49, 30-39, 15-29, <15 vs ≥60)	VDR
Best, 2002 (280) ³	5327	PCI	65	71	100	2.7	Death (251)	CG (50-69, 30-49, <30 vs ≥70)	VDW ⁴
Beddhu, 2002 (282) ³	8600	Coronary angiogram	62	68	100	3.2	MVE ^{††} (657), Death (1320)	MDRD (71-86, 57-70, <57 vs >86)	BLVDS
Nikolsky, 2004 (283) ³	1575	PCI + Diabetes	65	59	100	1 ^{††}	Death (125)	MDRD (<60 vs ≥60)	BVSW
Chen R, 2006 (288) ³	1609	Coronary angiogram	61	71	100	7 [†]	MVE	MDRD (<60 vs ≥60)	BDSWR
Hillis, 2006 (312)	2067	CABG	66 [†]	77	100	2.3 [†]	Death (382)	MDRD (45-59, 30-45, <30 vs ≥60)	
Mueller, 2004 (289) ³	1400	Angiogram for ACS	65	71	100	1.7	Death (158)	MDRD (60-74, 45-59, <45 vs ≥75)	D
Kontos, 2005 (286) ³	3074	ACS	57	50	100	1 ^{††}	Death (82)	MDRD (<60 vs ≥60)	BDV'S
Goldberg, 2005 (285) ³	1019	Acute MI	61	88	100	1 ^{††}	Death (259)	CG (30-59, <30 vs ≥60)	BD
Wright, 2002 (281) ³	3106	Acute MI		63	100	5 ^{††}	Death (138)	MDRD (60-89, ≤60 vs ≥90)	BVDS
Smith, 2006 (290) ³	44437	Acute MI	78	50	100	1 ^{††}	Death	CG (51-75, 35-50, <35 vs >75)	BVD
Ezekowitz, 2004 (284) ³	56652	HF inpatients	79	42	100	1 ^{††}	Death (15020)	MDRD (74-86, 67-73, 62-66, 55-61, 49-54, 42-48, 35-41, 25-34, ≤25 vs >87)	BDVSR
De Silva, 2006 (313)	955	CAD + HF	69	65	100	1 ^{††}	Death (21358)	CG (30-59, <30 vs ≥60)	LVDS
McAlister, 2004 (314)	754	HF outpatients	71	68	100	1.5 [†]	Death (145)	CG (<60 vs ≥60)	age, sex only
O'Hare, 2005 (287) ³	5787	HF outpatients	69 [†]	66	100	1 ^{††}	Death (203)	CG (60-89, 30-59, <30 vs ≥90)	BW
		Limb ischaemia	69	100	100	1 ^{††}	Death (1289)	MDRD (<30, 30-59 vs ≥60)	BVDR

See page 162 for notes and abbreviations

Table 5.2: Notes and abbreviations

¹ Adjustments are as follows; “B”, Blood pressure or history of hypertension; “L”, Plasma lipids or history of hypercholesterolaemia; “V”, presence or severity of vascular disease; “D”, history of diabetes; “S”, smoking status; “W”, weight or body mass index, “R”, race, ² Nested Case Control Study, ³ Studies in which the baseline blood sample was taken during an acute illness, ⁴ adjustments unclear, [†] Median, ^{††} Maximum, ^{*} Coronary Artery Disease only, ^{**} Myocardial Infarction only [§] Stroke only.

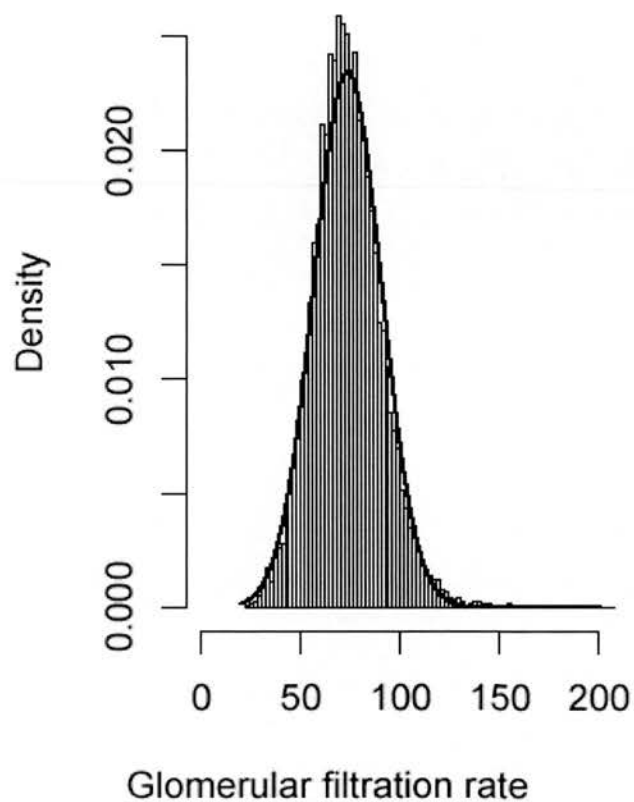
CVD, Cardiovascular Disease; FU, Follow-up; USA, United States of America; MVE, Major Vascular Events; MDRD, Modification of Diet in Renal Disease Study eGFR (ml/min/1.73m²); ARIC, Atherosclerotic Risk in Communities; CHS, Cardiovascular Health Study; BMES, Blue Mountain Eye Study; NHS, Nurses Health Study; CG, Cockcroft Gault creatinine clearance (ml/min); JPHC, Japan Public Health Centre-based study; MONICA, Monitoring Trends and Determinants on Cardiovascular Diseases, Augsburg; NHANES, National Health and Nutrition Estimation Survey; WHCP, Worksite Hypertension Control Program; ALLHAT, Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial; WOSCOPS, West of Scotland Coronary Prevention Study; CARE, Cholesterol And Recurrent Events; CAD, Coronary Artery Disease; MI, Myocardial Infarction; LIPID, Long-Term Intervention with Pravastatin in Ischaemic Disease; TAXUS-IV, Fourth Paclitaxel-eluting stent trial; BIP, Bezafibrate Infarction Prevention study; PEACE, Prevention of Events with ACE inhibition; HERS, Heart and Estrogen/progestin Replacement Study; VA-HIT, Veterans’ Affairs High-Density Lipoprotein Intervention Trial; TRACE, Trandolapril Cardiac Evaluation; Stent-PAMI, Stent Primary Angioplasty in Myocardial Infarction; VALLIANT, Valsartan in Acute Myocardial Infarction Trial; SAVE, Survival And Ventricular Enlargement; LVEF, Left Ventricular Ejection Fraction; SOLVED-P, Studies of Left Ventricular Dysfunction – Prevention; SOLVED-T, Studies of Left Ventricular Dysfunction – Treatment; HF, Heart Failure; PRIME II, Second Prospective Randomized study of Ibopamine on Mortality and Efficacy; DIG, Digitalis Intervention Group; MADIT-II, Multicenter Automatic Defibrillator Implantation Trial-II; CHARM, Candesartan in Heart Failure: Assessment of Reduction in Mortality and Morbidity; PCI, Percutaneous Coronary Intervention; CABG, Coronary Artery Bypass Graft; ACS, Acute Coronary Syndrome

Table 5.3: Results of cohort studies in which the relationships between blood cystatin c concentration, and either blood creatinine concentration or a creatinine based GFR estimate, and risk of death are assessed

Study	n	Population	Age mean	Men %	CVD %	FU mean	n deaths	exposure	Adjusted risk ratio for each cystatin c	creatinine	eGFR measure ^a
CHS, 2005 (166)	4637	Elderly	75	42	10	7.4 ^b	1316	Q1	1.0	1.0	1.0 ^d
								Q2	1.3 (0.9-2.0)	0.7 (0.6-0.9)	0.9 (0.7-1.1)
								Q3	1.9 (1.3-2.8)	0.8 (0.7-1.0)	0.9 (0.7-1.0)
								Q4	2.0 (1.4-2.9)	0.8 (0.7-1.0)	1.1 (0.9-1.3)
								Q5a	2.5 (1.6-3.8)	1.0 (0.8-1.3)	1.2 (0.9-1.6)
								Q5b	2.7 (1.8-4.1)	1.0 (0.7-1.2)	1.3 (1.0-1.6)
								Q5c	2.8 (1.9-4.3)	1.5 (1.2-1.9)	1.8 (1.4-2.2)
								Q1	1.0	1.0	
								Q2	1.7 (1.2-2.5)	1.0 (0.7-1.4)	
Health ABC, 2006 (315)	3044	Elderly	73	49	20	6 ^c	557	Q3	1.5 (1.1-2.2)	1.0 (0.7-1.3)	
								Q4	1.5 (1.0-2.1)	1.1 (0.8-1.6)	
								Q5	2.2 (1.5-3.1)	1.2 (0.9-1.6)	
								per SD increase	1.8 (1.4-2.4)	1.5 (1.2-2.0)	0.6 (0.4-1.0) ^e
FINN-AKVA, 2007 (316)	480	Acute heart failure	75	50	100	1 ^c	122				

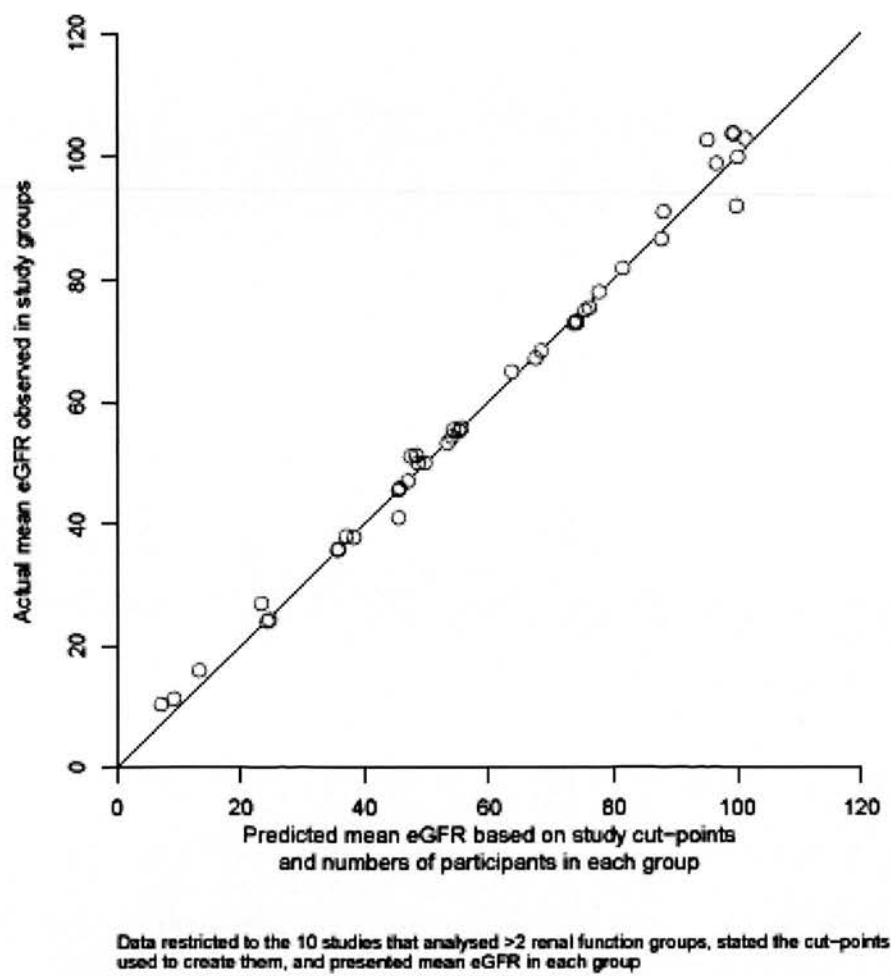
^a All risk ratios are adjusted for at least age and sex. Additional adjustments included co-morbidity (diabetes and cardiovascular disease), left ventricular hypertrophy, blood C-reactive protein, haemoglobin concentrations in the CHS, and co-morbidity (diabetes, cardiovascular disease and hypertension), blood concentration of lipids and inflammatory markers (IL-6, CRP, TNF- α), body mass index, race and level of education in the Health ABC study. Risk estimates in the FINN-AKVA study were adjusted for blood pressure, haemoglobin concentration and N-terminal brain natriuretic peptide. In each study the same model of adjustment was used for each GFR measure, ^b Median, ^c Maximum, ^d GFR estimated by the MDRD formula the quintiles are arranged in reverse order to facilitate comparison with other GFR measures (i.e. the reference group is the highest quintile of eGFR), ^e GFR estimated by the Cockcroft-Gault formula.
n, number of individuals; CVD, cardiovascular disease; FU, follow-up; GFR, glomerular filtration rate; CHS, Cardiovascular Health Study; Health ABC, Health Aging and Body Composition study; FINN-AKVA, Finnish Acute Heart Failure Study; Q, quintile (in the cardiovascular health study the top quintile is further divided into 3); SD, standard deviation

Figure 5.1: Baseline eGFR among 20 536 participants in the Heart Protection Study



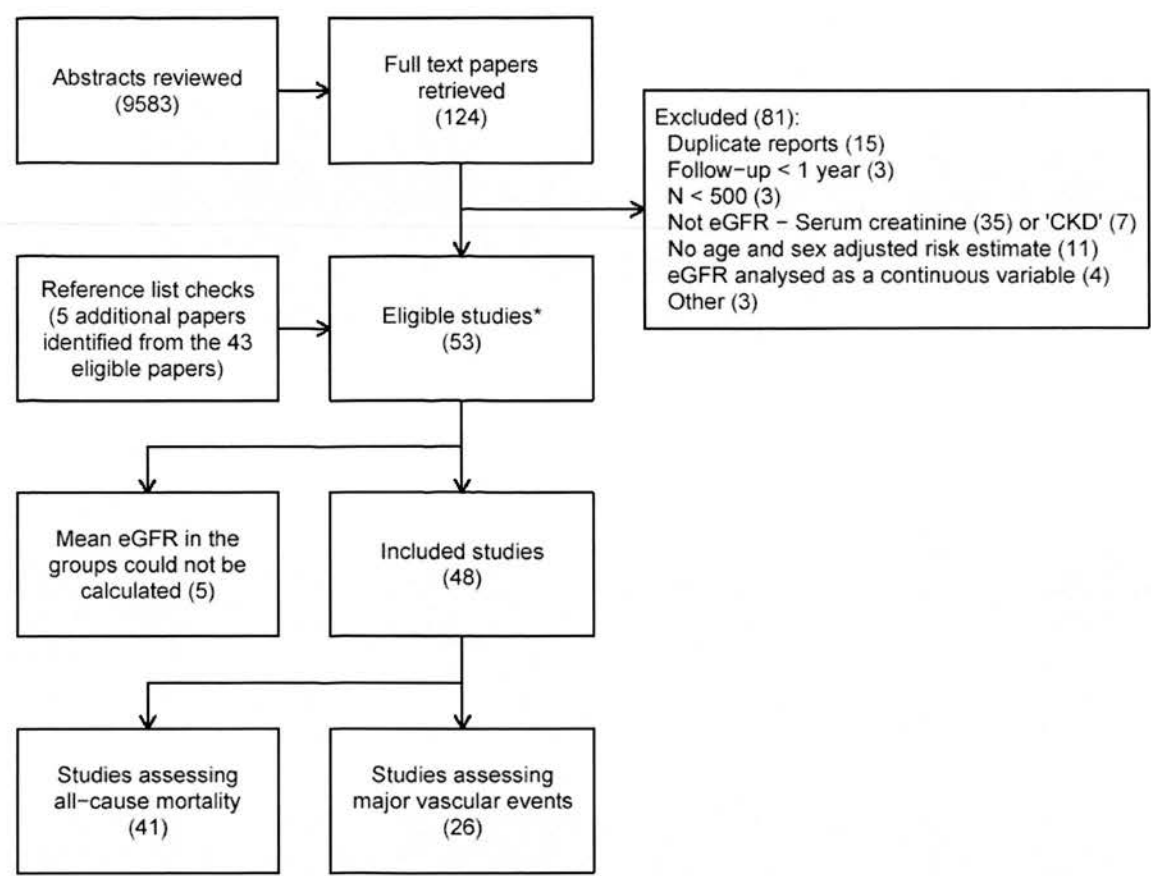
eGFR (in ml/min/1.73m²) was estimated using the abbreviated MDRD equation

Figure 5.2: Comparison of actual vs estimated mean eGFR levels in the comparison groups



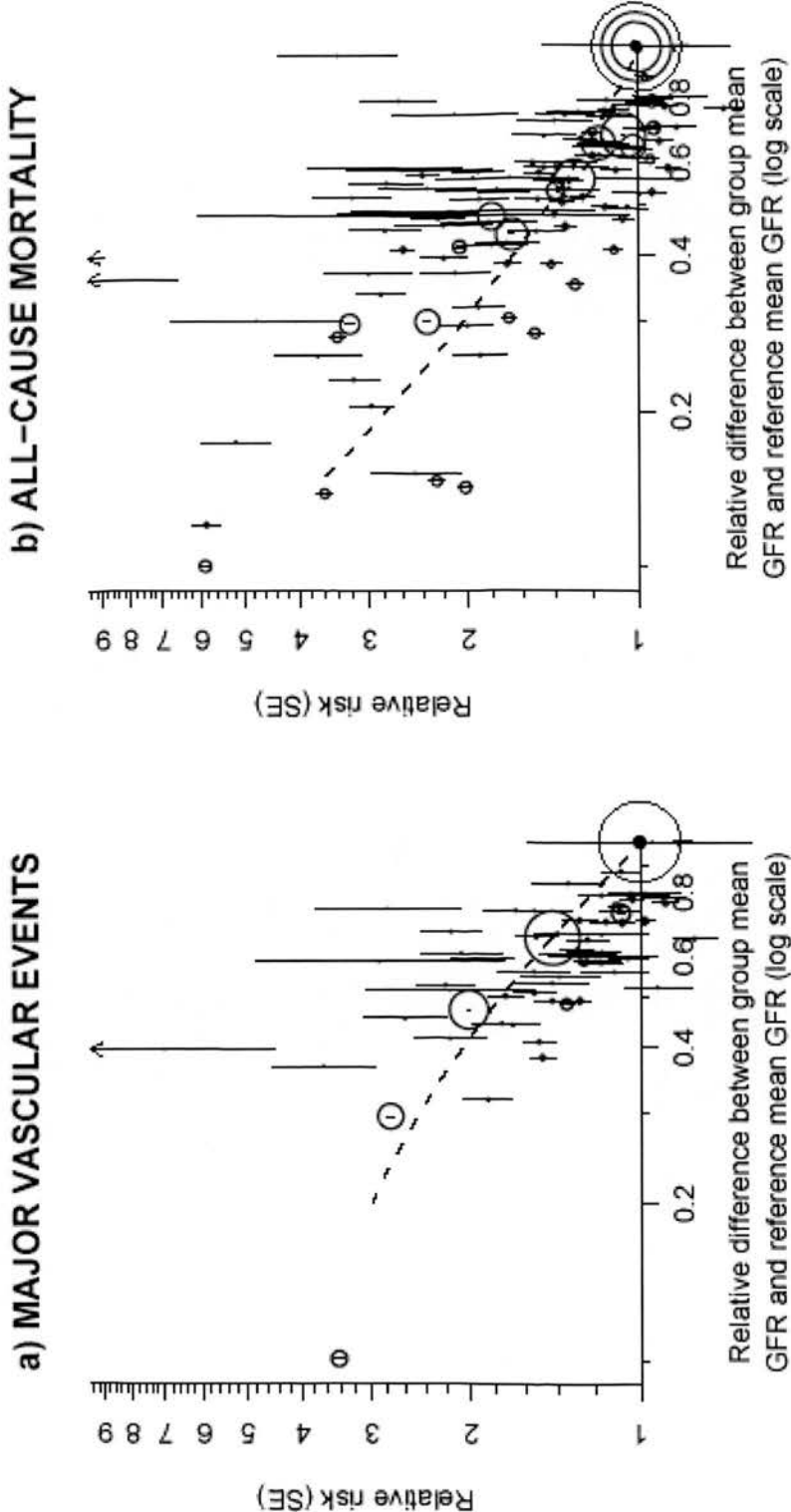
eGFR (in ml/min or ml/min/1.73m²) was estimated in the published studies using either an MDRD equation or the Cockcroft and Gault formula (Table 5.2)

Figure 5.3: Results of the literature search



eGFR: estimated glomerular filtration rate; CKD: chronic kidney disease
* Count of the number of studies, not the number of papers (different subsets of some study populations appear in different papers while some papers present data on more than one study)

Figure 5.4: Dose-response relationship between proportional reductions in eGFR and risk of a) major vascular events and b) all cause mortality

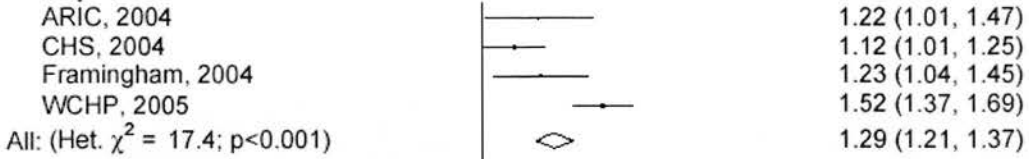


Relative risks together with approximate floated standard errors are shown on the log scale. The area of each plotting symbol is proportional to the amount of statistical information (i.e. it is inversely proportional to the variance of the log odds ratio). Best inverse variance weighted linear or quadratic fits are superimposed depending on the statistical significance of the quadratic term. The vertical lines through the points represent standard error bars.

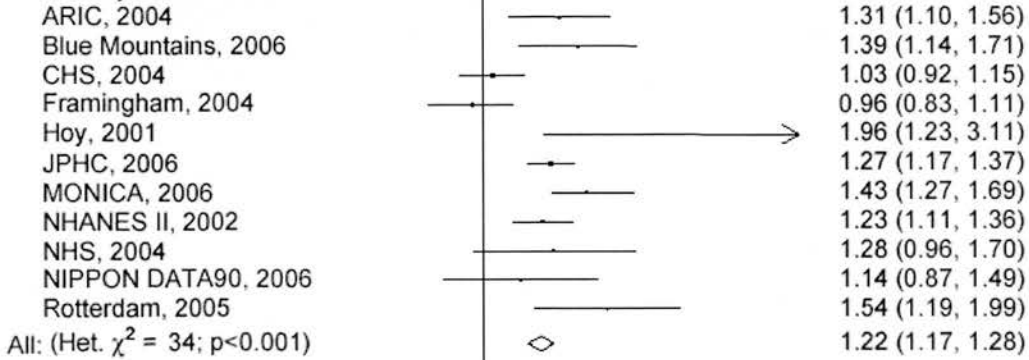
Figure 5.5: Risk of major vascular events associated with a 30% lower eGFR

Prospective cohort studies

With prior CVD

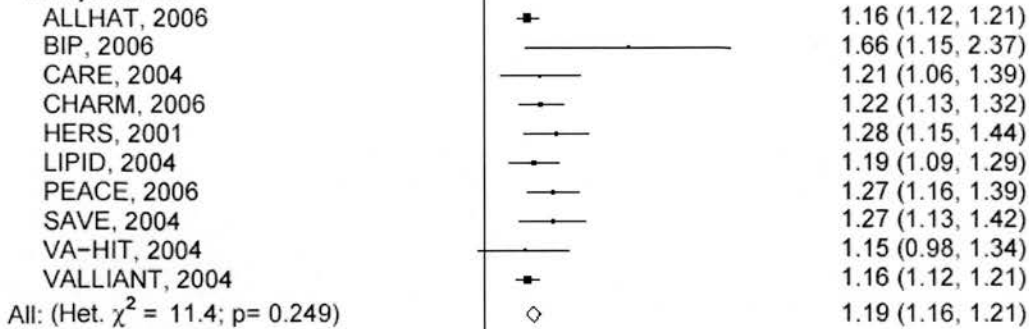


Without prior CVD



Randomised controlled trials

With prior CVD

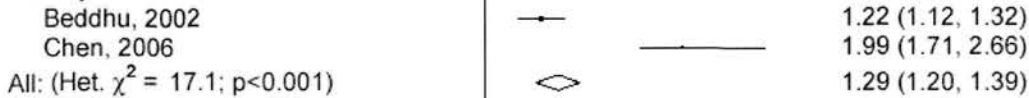


Without prior CVD



Routine data

With prior CVD



Without prior CVD



RR (95% CI) associated with
a 30% lower eGFR

Figure 5.6: Risk of all cause mortality associated with a 30% lower eGFR

Prospective cohort studies

With prior CVD

ARIC, 2004

CHS, 2004

Framingham, 2004

WCHP, 2005

All: (Het. $\chi^2 = 16.7$; $p < 0.001$)

Without prior CVD

ARIC, 2004

CHS, 2004

Framingham, 2004

Hoy, 2001

JPHC, 2006

MONICA, 2006

NHANES II, 2002

NIPPON DATA90, 2006

All: (Het. $\chi^2 = 58.3$; $p < 0.001$)

Randomised controlled trials

With prior CVD

CARE, 2004

CHARM, 2006

DIG, 2004

LIPID, 2004

MADIT-II, 2006

PEACE, 2006

PRIME II, 2000

SAVE, 2004

SOLVED (P), 2000

SOLVED (T), 2000

Stent-PAMI, 2003

TAXUS-IV, 2005

TRACE, 2002

VALLIANT, 2004

All: (Het. $\chi^2 = 13.7$; $p = 0.395$)

Without prior CVD

WOSCOPS, 2004

Routine data

With prior CVD

Beddhu, 2002

Best, 2002

Chen, 2006

de Silva, 2006

Ezekowitz, 2004

Goldberg, 2005

Hillis, 2006

Kontos, 2005

McAlister, 2004

Mueller, 2004

Nikolsky, 2004

O'Hare, 2005

O'Hare, 2006

Smith (HF), 2006

Smith (MI), 2006

Wright, 2002

All: (Het. $\chi^2 = 151.7$; $p < 0.001$)

Without prior CVD

Go, 2004

O'Hare, 2006

All (Het. $\chi^2 = 63.8$; $p < 0.001$)

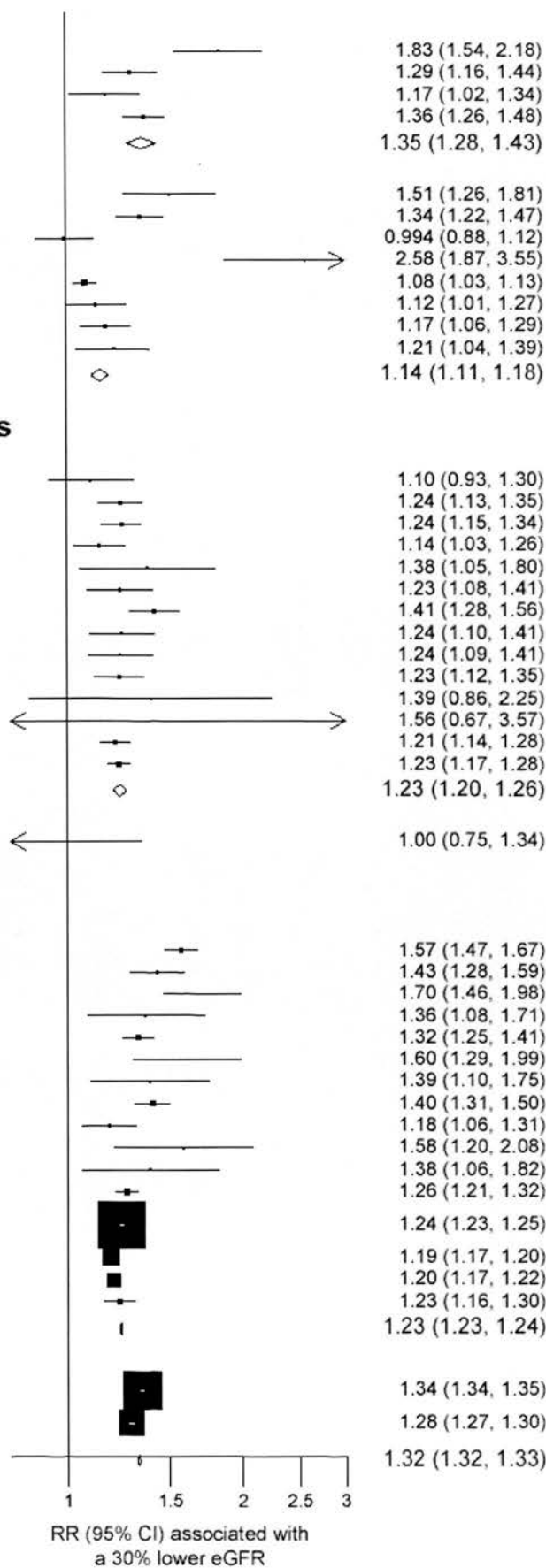


Figure 5.7: Relationship between proportional reductions in eGFR stratified by study population type

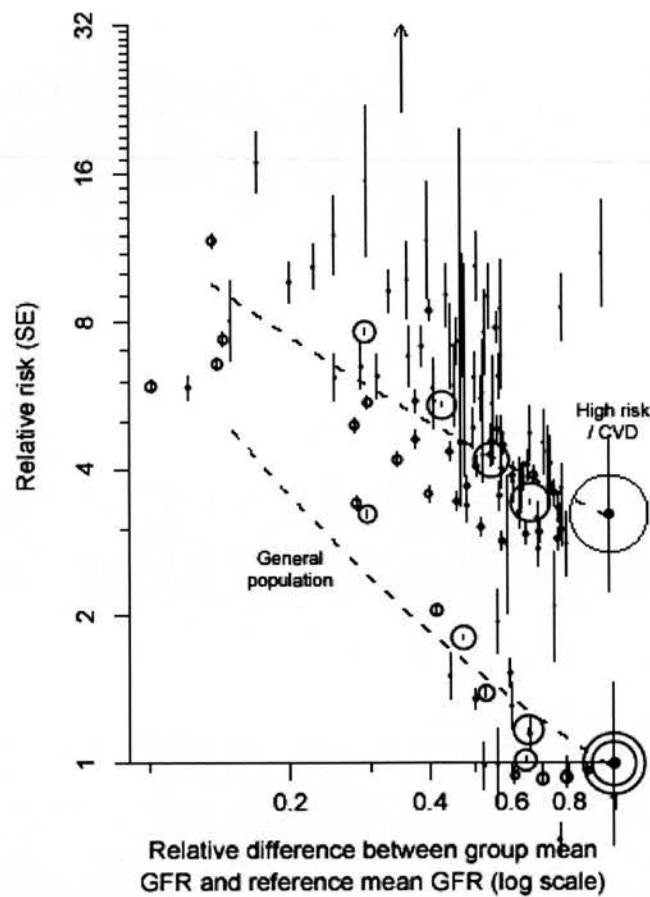


Figure 5.8: Age specific association between eGFR and all cause mortality in the Veterans' Affairs study

Without prior CVD

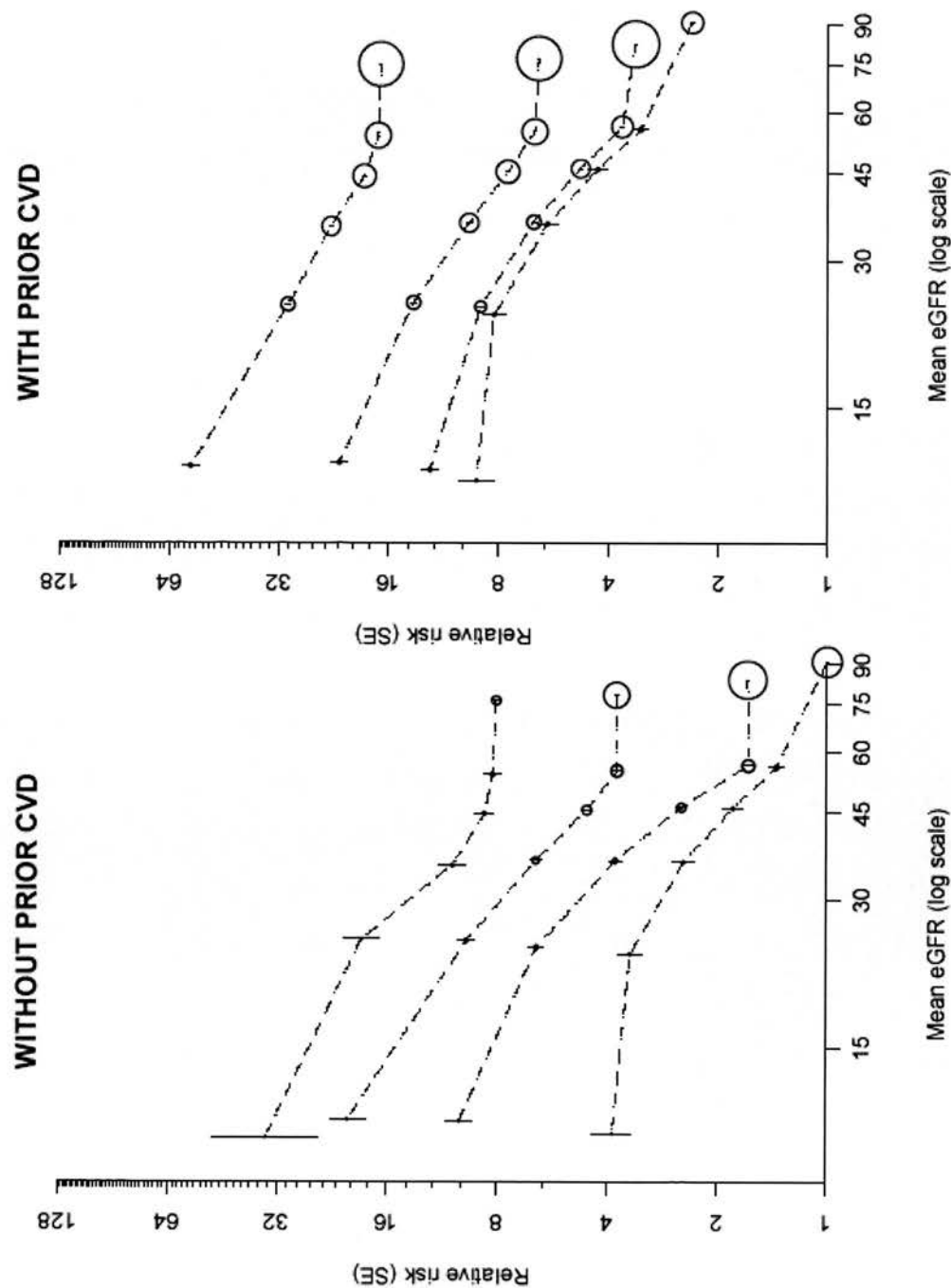
18-44 years		1.48 (1.40, 1.57)
45-54 years		1.43 (1.39, 1.46)
55-64 years		1.39 (1.36, 1.43)
65-74 years		1.31 (1.28, 1.33)
75-84 years		1.24 (1.22, 1.27)
85-100 years		1.18 (1.13, 1.24)

With prior CVD

18-44 years		1.32 (1.23, 1.42)
45-54 years		1.34 (1.31, 1.37)
55-64 years		1.32 (1.30, 1.34)
65-74 years		1.27 (1.26, 1.29)
75-84 years		1.23 (1.22, 1.24)
85-100 years		1.17 (1.16, 1.18)

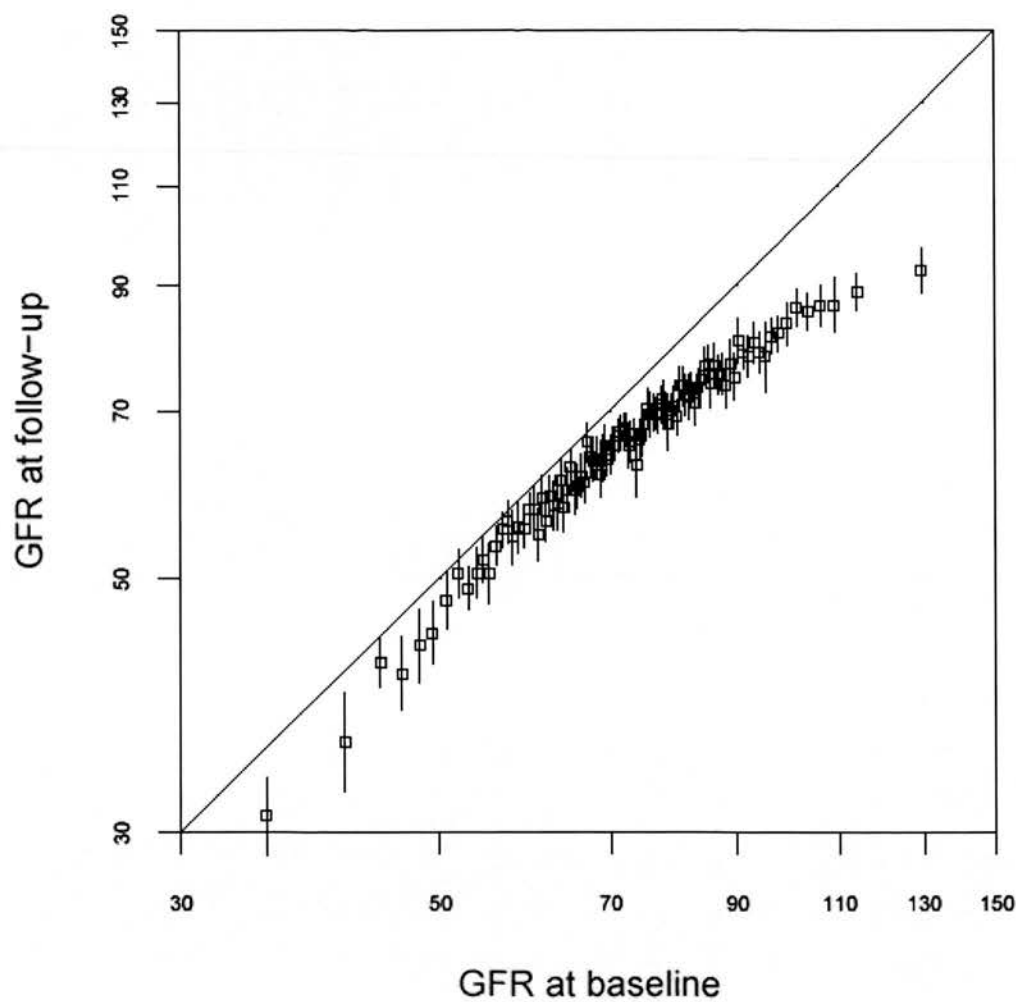
1 1.5 2 2.5 3
RR (95% CI) associated with
a 30% lower eGFR

Figure 5.9: Association between eGFR and all cause mortality in the Veterans' Affairs study, at ages 18-54, 55-74, 75-84 and 85-100



In the Veteran's Affairs study, eGFR (in ml/min/1.73m²) was estimated using the abbreviated MDRD equation

Figure 5.10: Mean follow-up eGFR by percentile of the baseline distribution among 7697 patients allocated placebo in the MRC/BHF Heart Protection Study (follow-up 4-5 years later)



eGFR (in ml/min/1.73m²) was estimated using the abbreviated MDRD equation

Conclusions

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6.1 Summary of results

6.1.1 Measurement of GFR in large scale epidemiological studies

This thesis describes the current methods by which GFR can be measured or estimated (Chapter 1). Direct methods, in which GFR is assessed from the total plasma clearance, or renal clearance, of an exogenous tracer compound using timed blood samples, with or without urine collections, are accurate but time consuming and not suitable for large scale epidemiological studies.

Indirect methods, in which GFR is estimated from a single measurement of the blood concentration of an endogenous substance such as creatinine or cystatin c, are simple to perform, but existing methods have two key limitations:

First, GFR estimated from blood creatinine and cystatin c concentrations are heavily dependent on the laboratory method used, meaning that large biases can occur when the laboratory methods and calibration are different to that used to develop the prediction equations (161;167).

Second, and more importantly, because of the reciprocal relationship with GFR and inter-personal differences in rate of production, among healthy individuals any blood concentration of creatinine or cystatin c is consistent with a wide range of the range of possible GFR values (104;156). Therefore, for any individual, GFR estimated by blood

creatinine or cystatin c concentration may be widely discrepant from their 'true' GFR (Chapters 1 and 2).

A novel method, in which total plasma iohexol clearance is measured using dried capillary blood spots collected by study staff 2, 3 and 4 hours after intravenous injection, accurately assesses GFR compared to traditional iohexol clearance using 3 timed plasma samples (Chapter 2). Using only 2 capillary blood spots collected at 2 and 4 hours does not lead to loss of accuracy (Chapter 2). This method has two key advantages for use in large scale epidemiological studies: First, the method remains accurate when the dried capillary blood spots are sent through the post, meaning that expensive transfer of refrigerated samples or training and validation of local laboratories would not be necessary for multi-centre studies (Chapter 2). Second, the blood spots could potentially be completed by participants themselves at home substantially reducing the amount of clinic time required. However, a cross-sectional feasibility study, in which volunteers were asked to undertake the blood spot iohexol clearance (BSIC) procedure collecting 2 timed capillary blood spots themselves after the clinic (Chapter 3), raised a number of problems associated with this method. One hundred percent of eligible participants were successfully trained to collect suitable capillary blood samples during the clinic visit, of whom 96% returned 2 analysable capillary blood spots and 90% found the blood spot collection procedure acceptable. However, 21% of participants returned small or 'poor quality' blood spots. In addition, there was statistical evidence the the sampling time had not been accurately recorded by all participants and, more importantly, there was evidence that large discrepancies between the actual sampling time and the recorded

sampling time had occurred. Although, when the blood spots are collected by study staff, a simulated situation equivalent to one third of individuals misreporting the sample time by 15 minutes or more had little effect on the accuracy of the blood spot iohexol clearance method, larger discrepancies between actual and recorded sampling times would substantially reduce test accuracy (Chapter 2). Improvements in the biochemical methods to make use of small or poor quality spots, development of an automated timing device and further validation of the accuracy of the blood spot iohexol clearance technique when samples are collected outside the clinic are needed before using this method can be used in large scale studies.

6.1.2 Relationship between measures of GFR and cardiovascular risk factors

In cross-sectional studies, chronic kidney disease is associated with hypertension (5;24), a pro-atherogenic lipid profile (small dense LDL particles (317;318), lower HDL-cholesterol (5;49) and increased blood triglycerides (5;43), higher blood concentrations of C-reactive protein (52;54), fibrinogen (54;56) and homocysteine (27;240), while increased body fat is an independent risk factor for the development of chronic kidney disease in prospective studies (256;257). Animal studies suggest the association between chronic kidney disease and hypertension (33) and dyslipidaemia (32) may be causal.

In a cross-sectional study of individuals with preserved GFR (mean \pm standard deviation GFR 103.1 ± 28.3 ml/min/1.73m²), in which GFR was assessed by blood spot iohexol clearance, the MDRD formula and estimated from serum cystatin c concentration, one or more of the GFR measure were positively correlated with blood HDL-cholesterol and

apolipoprotein A₁ concentrations, and were inversely correlated with blood concentrations of triglyceride, CRP, fibrinogen, and homocysteine (Chapter 4). No correlation was observed between any of the GFR measures and diastolic blood pressure, systolic blood pressure, or blood concentration of total-cholesterol, LDL-cholesterol or apo-lipoprotein b in this sample, although the greater use of antihypertensive and lipid lowering medication among those with lower GFR limits interpretation of these findings. The magnitude of the change in these cardiovascular risk factors accompanying a 30ml/min1.73m² lower GFR could be associated with a small but potentially clinically relevant increase in risk of cardiovascular disease. This study considered how these relationships might be affected by the method used to assess GFR. Surprisingly, GFR measured by blood spot iothexol clearance (BSIC) was not more strongly related to cardiovascular risk factors than that estimated from the MDRD formula, although this is probably the result of inaccuracies in the BSIC method given the methodological issues raised in chapter 3. GFR estimated from blood cystatin c concentration tended to be more strongly correlated with blood lipids and inflammatory markers than GFR measured by the other two methods. Measures of body fat were strongly correlated with GFR estimated from cystatin c concentration, but not with either GFR estimated from the MDRD equation or measured using blood spot iothexol clearance raising the possibility that body fat might be related to cystatin c production.

6.1.3 Relationship between GFR and risk of cardiovascular disease and death

No large scale prospective cohort studies have measured GFR directly. In a quantitative meta-analysis of prospective studies assessing the relationship between GFR estimated

from serum creatinine concentration (eGFR) and risk of death or cardiovascular events (which included 4 061 003 and 1 372 820 individuals from 41 and 26 cohort studies for the outcome of death and major vascular events respectively), a proportional reduction in baseline eGFR was consistently associated with an increase risk of both outcomes, whether the data for the study was collected as part of (i) a prospective cohort study (ii) a randomized trial, or (iii) routine clinical or administrative practice (routine data sources) (Chapter 5). For major vascular events this relationship was approximately log-linear, whereas for death there was evidence of non-linearity, where small reductions in eGFR of less than 20% were not associated with increased risk. Overall, a 30% lower eGFR was associated with a 20-30% increase in risk of both death and vascular events depending on the type of study examined, although significant heterogeneity was observed between the results of the individual prospective cohort studies and routine data sources for both outcomes, which was not explained by the presence of prior vascular disease. One large routine data source provided data on the risk of death associated with lower eGFR separately by age group. Although the relative impact of eGFR was larger at younger age groups the absolute relevance of eGFR to risk of death was greater at older ages and among those with prior vascular disease. In two large routine data sources, which together contributed 85% of the available deaths, reduction in GFR was not associated with increased risk until eGFR was $< 60 \text{ ml/min/1.73m}^2$, suggesting that the overall 20-30% increased risk of death associated with a 30% lower eGFR may be an underestimate among those with chronic kidney disease. However, the mean eGFR in the reference groups was around $84 \text{ ml/min/1.73m}^2$ therefore this meta-

analysis is not able to properly assess the dose response relationship between lower eGFR and risk of death and vascular events across the range of GFR in the population.

Since GFR was not measured in these studies, but estimated from blood creatinine concentration, misclassification between eGFR categories will occur. When the MDRD formula is used to classify individuals into K / DOQI CKD stages (≥ 90 , 60-89, 30-59, 15-29, <15 ml/min/1.73m² (16)), nearly 30% of individuals are misclassified (21). This degree of misclassification would lead to substantial distortion of the real relationship between true GFR and risk of death and cardiovascular events when eGFR is used (170). Large scale studies in which GFR is measured directly are needed to properly assess the impact of GFR on important outcomes such as death and cardiovascular morbidity, and to reliably assess the interaction between GFR and cardiovascular risk factors.

6.2 Implications for observational epidemiology

In epidemiological studies, it may be important to assess GFR for 2 reasons:

First, one might wish to examine the relationship between GFR as an exposure variable and a particular outcome. Second, assessment of GFR would allow adjustment for GFR in the examination of other exposures which correlated with GFR, for example blood homocysteine concentration. The method used to measure GFR will affect both these aims. When GFR is examined as an exposure, GFR estimated from blood creatinine or cystatin c concentration may lead to an underestimate of the strength of the relationship because of misclassification. While GFR estimated from the MDRD or Cockcroft Gault formula is more informative than serum creatinine alone when assessing an individual's GFR in clinical practice (16), an analysis of a cohort study adjusted for age, sex and race,

eGFR provides no more information than one which uses blood creatinine concentration alone. Since the accuracy of cystatin c is similar to that of MDRD eGFR without the inclusion of demographic variables, it is more useful in epidemiology. However, both measures predict GFR poorly among healthy individuals (Chapters 1 and 2). In cohort studies examining the relationship between GFR and a particular outcome, misclassification between GFR categories because of this inaccuracy will tend to bias the results toward the null (170), while when eGFR is used to adjust for GFR, residual confounding could remain.

In addition, non renal influences on the concentration of endogenous marker may introduce specific biases. Blood cystatin c concentrations are strongly correlated with body mass index, waist circumference and percentage body fat measured by single frequency bioimpedance, when no correlation is observed between these measures of body composition and either GFR measured by blood spot iothexol clearance or GFR estimated by the MDRD formula (Chapter 4). In a large cross-sectional survey, cystatin c was related to weight after adjustment for measured creatinine clearance (141). These findings raise the possibility that body fat might be related to cystatin c production, which could result in bias when cystatin c is used to assess the relationship between GFR and cardiovascular disease.

6.3 Implications for clinical practice

GFR is rarely measured directly in clinical practice and is usually assessed from measurement of serum creatinine concentration. GFR is usually assessed in clinical practice in order to detect acute renal failure in acutely unwell patients, appropriately

adjust the dose of drugs which are excreted via the kidney, or to identify individuals at risk of developing end stage renal failure. In 2005 the Department of Health's National Service Framework for Renal Services recommended that eGFR is used routinely in clinical practice in preference to serum creatinine concentration alone. Identification of individuals with chronic kidney disease based on estimated GFR (16), and assessment of cardiovascular risk factors among these individuals, became a Quality Outcomes Framework target for general practitioners in 2006. Since eGFR can be widely discrepant from true GFR and, in healthy populations, may systematically underestimate 'true' GFR (Chapters 1 and 2), clinicians should be aware of the limitations using this test to classify individuals as having a chronic disease.

This research would support the concept of reduced GFR as an independent risk factor for cardiovascular disease (Chapter 5), although a number of substantial uncertainties remain: The strength of the true relationship between GFR and risk of cardiovascular events across the range of population GFR values remains poorly understood, if this association is causal is unknown, and, if it is, how it is mediated and therefore how might it be ameliorated is uncertain. Current practices identify a group of individuals with reduced eGFR who could be recruited to future randomized clinical trials.

6.4 Future research

In assessing the hypothesis that small reductions in GFR result in clustering of cardiovascular risk factors leading to increase risk of cardiovascular disease 3 areas of further research should be considered.

First, large scale prospective cohort studies of healthy individuals in which GFR is measured directly are needed in order to properly assess the real relationship between ‘true’ GFR and cardiovascular outcomes across the population range of GFR. Potential mechanisms for such an association could be explored by assessing the effect of adjusting for cardiovascular risk factors which might be on the causal pathway such as blood pressure, blood lipid concentrations, markers of inflammation and blood homocysteine concentration. Blood spot iothexol clearance has potential for use in such studies since, if participants can complete the blood sampling at home, it would substantially reduce the clinic time required compared to traditional direct GFR methods, and would allow transportation of samples at room temperature to a reference laboratory. However, before this method can be used in large scale studies, biochemical methods must be improved to enable accurate determination of iothexol concentration in small and poor quality blood spots, and a method of automatically recording the sampling time, perhaps with a combined timing and sampling device, must be developed. A large population sample in which GFR is assessed accurately would also be important in developing better indirect measures of GFR by enabling;

- Reliable assessment of the non-renal influences on blood cystatin c concentration,
- Development of formulae which include demographic variables, creatinine and cystatin c for potential use in clinical practice,
- Storage of blood to allow assessment of new endogenous GFR markers which may be more accurate than cystatin c

Second, assessment of cardiovascular risk factors pre and post nephrectomy in healthy kidney donors might help to determine the causal direction of the relationship between lower GFR and cardiovascular risk factors.

Third, large scale clinical trials of cardio-protective interventions among individuals with reduced GFR are needed to establish which interventions might prevent the excess cardiovascular disease in this population.

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Publications

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Permission has been obtained from all authors to include data from these publications in this thesis.